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# PHYSIOLOGICAL STUDIES ON AN OBLIGATE PSYCHROPHILE, *MICROCOCCUS CRYOPHILUS*, AND ITS MESOPHILIC MUTANTS

by

©

PHANG-CHENG TAI

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

PHYSIOLOGICAL STUDIES ON AN OBLIGATE
PSYCHROPHILE, MICROCOCCUS CRYOPHILUS,
AND ITS MESOPHILIC MUTANTS

submitted by Phang-cheng Tai in partial fulfilment of the requirements for the degree of Master of Science.

# ABSTRACT

Several mutants with elevated maximum growth temperature (MGT) were developed from an obligate psychrophile, *Micrococcus cryophilus* ATCC 15174, by means of UV-irradiation. Two of the mutants, T8 and M19 were demonstrated to be genetically related to the parent by the production of transformants. The mutants lost the ability to grow well at 0°C and showed changes in metabolism while acquiring the ability to grow at elevated temperatures. The significance of the mutants and the transformants is discussed.

Heat resistance, DNA denaturation temperature, solute-transporting and energy-yielding systems were shown to be unrelated to MGT in this organism.

Endogenous respiration and temperature characteristic ($\mu$) for substrate oxidation were found to be unsuitable as a means of characterizing mesophiles and psychrophiles. $Q_{10}$ of substrate oxidation, with reference temperatures set at 10°C and 30°C, is suggested for this purpose, the mesophile having a higher value than the psychrophile. The use of $\mu$ for this same purpose is discussed.

Studies of amino acyl-t-RNA synthetase activity and incor-

poration of $^{14}C$-leucine into protein showed that protein synthesis is not the primary factor in determining the heat-sensitivity of *M. cryophilus*.

It was found that RNase in cell extracts and RNase,purified 120-fold from *M. cryophilus*,has a constant activity below 25°C, near the MGT of this organism, and a greatly increased activity above this temperature. RNA polymerase activity showed a decrease and DNase an increase above 25°C. A possible relationship between these findings, interaction of protein and RNA synthesis, and a mechanism and "lethal" action of "activated" RNase activity is discussed.

It is suggested that RNA, and possibly DNA, and related enzymes are the primary factors in determining heat-sensitivity of *M. cryophilus*, and that the action of RNA polymerase and RNase on RNA, probably t-RNA, is the basis of MGT. The mutant M19 probably has the same basis of heat-sensitivity and MGT as its parent.

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# GENERAL INTRODUCTION

Micro-organisms are generally divided into thermophiles, mesophiles and psychrophiles on the basis of the temperature ranges in which they can grow. The importance of psychrophilic micro-organisms to the dairy and food industry has increased tremendously during the past decade, mainly because of the increasing use of refrigeration. Psychrophiles are also of academic interest, since they possess the yet unexplained phenomenon of growth at low temperature. The subject of psychrophilic bacteria has been reviewed in relation to food by Ingram (1951), and Mossel and Ingram (1955); in relation to dairying by Davis (1951), Doetsch and Scott (1951) and Witter (1961); and in relation to general bacteriology by Ingraham and Stokes (1959), and Farrell and Rose (1965 and 1967).

The psychrophilic micro-organisms are distinguished from others by their ability to grow at low temperature, now generally accepted as "micro-organisms that grow well at 0°C within 2 weeks, or have a generation time within 48 hours at 0°C" (Ingraham and Stokes, 1959). The fundamental problem is why these organisms grow at low temperature, or, from another point of view, why psychrophilic micro-organisms cannot grow at elevated temperature.

## I. PHENOMENON OF GROWTH AT LOW-TEMPERATURE

As the temperature is lowered there are some cellular activities which have been shown to increase or to qualitatively appear. Jordan and Jacobs (1947) concluded that *Escherichia coli* utilized nutrients more efficiently at 15°C than at 35°C for the formation of cellular material. Thiel (1940a, 1940b) showed an increase in the production of certain acids when the incubation temperature was reduced. Also, the efficiency of fermentation as an energy source for growth is greater at low temperature (Dorn and Rahn, 1939). Pigment production has long been considered a prominent phenomenon. It has been observed that a flavobacterium produced a more brilliant yellow pigment at 5°C than at 20°C (Hess, 1934), and that the pigment formation of *Pseudomonas nigrifaciens* was greater at 4°C than at 15°C and absent at 25°C (White, 1940). Similar results were obtained by Hiscox (1936).

The minimum temperatures for some metabolic activities are below the minimum temperature for growth. Thus, most mesophilic micro-organisms probably respire endogenous reserves at temperatures well below the minimum temperature for growth (Rose and Evison, 1965), and *Streptococcus lactis* has been reported to produce lactic acid at 0°C, although it does not grow at this temperature (Borgstrom, 1962).

Spores of many bacilli germinate at 5°C, which is well below minimal temperature of growth (Williams et al., 1957).

Data reported by Hess (1934) showed that although Pseudomonas fluorescens grow most rapidly at 20°C, the largest crop of bacteria was formed at 5°C. A similar observation was made by Dorn and Rahn (1939) with Strep. lactis. However, Sinclair and Stokes (1963) have shown that equal size of cells could be obtained at higher temperature when the cultures were vigorously aerated.

Sherman and Albus (1923) first reported the phenomenon of "cold shock". When certain Gram-negative bacteria in the exponential phase of growth that had been grown at 30° to 40°C were suspended in a diluent and then rapidly cooled to 0 to 5°C, a large proportion of the organisms was killed. This loss of viability was accompanied by release from the bacteria of endogenous solutes including nucleotides, amino acids and adenosine triphosphate into the medium (Strange and Dark, 1962).

A change in cell composition which could well be important in determining the ability of micro-organisms to grow at low temperature, is the increase in the proportion of unsaturated fatty acids in the cellular lipids as the temperature of incubation decreases (Kates and Baxter, 1962). Similar results have been reported by Marr and

Ingraham (1962).

The effects of low temperatures on individual metabolic activities of micro-organisms reflect differences in the synthesis and activities of individual enzymes at the various temperatures. Nashif and Nelson (1953) showed that Ps. fragi produced a lipase preferentially at low temperatures and not at all at 30°C, although the optimum temperature for activity of the enzyme was 40°C. The synthesis of protease by Ps. fluororescens, β-galactosidase and tryptophanase in E. coli were found to be similarly affected by low temperature (Peterson and Gunderson, 1960; Horiuchi and Novick, 1961; Ng and Gartner, 1963).

The nutritional requirements of some micro-organisms vary with the temperature at which the organisms are grown. Maas (1961) described a mutant strain of E. coli which synthesized ornithine carbamoyl transferase at 37°C, but not at 25°C, and therefore required arginine at the lower but not at the higher temperature. A diminished requirement for a growth factor at a low temperature has been reported by Barnett and Lilly (1948). O'Donovan et al. (1965) reported a mutant of E.coli requiring histidine only at temperatures below 20°C.

Since psychrophiles are distinguished from mesophiles by their ability to grow well at 0°C, it is natural to inquire whether mesophiles can become "adapted" to grow at 0°C. There are a number

of reports of attempts, but, with one or two exceptions (e.g. Kluyver and Baars, 1932), none of them has claimed to have obtained an appreciable lowering of the minimum temperature for growth of the organisms. However, Azuma _et al_. (1962) by using ultraviolet irradiation were able to isolate psychrophilic mutants from mesophilic organisms.

## II. DIFFERENCES BETWEEN PSYCHROPHILES AND MESOPHILES

The main difference between psychrophilic and mesophilic micro-organisms is the temperature range of growth. Ingraham (1958) reported that psychrophiles have an 8 to 10°C lowering of minimum growth temperature. Foter and Rahn (1936) observed that, since growth and respiration consist of chemical reactions, these processes should continue, though at a greatly decreased rate, until the medium in which the micro-organisms are suspended freezes solid. Although this behaviour is found with psychrophilic micro-organisms, it is not characteristic of mesophiles.

Brown (1957) reported that the oxygen uptake with glucose by a psychrophile had a lower temperature characteristic (slope of an Arrhenius plot) than that of mesophilic _Ps. aeruginosa_, and showed that the psychrophile was less sensitive to changes in temperature. Ingraham (1958) compared the effect of incubation temperature on the

generation times of a psychrophilic and a mesophilic bacterium, and showed that the psychrophile had a lower temperature characteristic ($\mu$) as compared with the mesophiles. In a subsequent study, Ingraham and Bailey (1959) reported that the temperature coefficients ($Q_{10}$) of the tested psychrophiles for glucose oxidation, acetate oxidation and formate oxidation were lower than those for the tested mesophiles. However, when the activities of these three dehydrogenase were measured, the activation energies were very similar. This suggests that the difference in temperature responses between psychrophiles and mesophiles might be associated with the integrity of cellular organization. Burton and Morita (1965) have shown that the malate dehydrogenase from the psychrophile, Vibrio marinus, has an activation energy only half that of the corresponding enzyme from a mesophilic strain of Escherichia coli. However, Shaw (1967), and Hanus and Morita (1967) both reported that the psychrophiles cannot be distinguished from the mesophiles on the basis of the temperature characteristic, which was found to be the same for both types.

Baxter and Gibbons (1962) found that a psychrophilic strain of Candida respired endogenous reserves at a greater rate than the mesophiles at all temperatures up to 30°C, and that, although the psychrophile oxidized glucose at an appreciable rate, even at 0°C, virtually no exogenous substrate was oxidized by the meso-phile at temperatures

below 5°C. The data suggested that only the psychrophile but not mesophile was able to transport sugars into the cell at temperatures below 5°C. Kates and Baxter (1962) found no difference of the total extractable lipids in mesophilic and psychrophilic strains of Candida, but that of the psychrophile contained a higher percentage of unsaturated fatty acids. Also, the psychrophiles contained trienoic acid and linolenic acid which could not be detected in the mesophile. Similar results have been reported by Marr and Ingraham (1962), Kates and Hagen (1964). Sultzer (1961) observed that a psychrophilic pseudomonad exhibited significantly lower $\mu$ value while oxidizing octanoate at much higher rates through the same temperature range than mesophiles. It has been suggested that protein carrier molecules differ in molecular architecture in mesophiles and psychrophiles (Farrell and Rose, 1965). However, Cirillo et al. (1963) showed that sugar transport in both the psychrophile and mesophile used met the criteria for carrier mediated facilitated diffusion.

## III. THE BASIS OF MINIMUM TEMPERATURE OF GROWTH

Baxter and Gibbons (1962) suggested that the main factor determining the minimum temperature for growth of micro-organisms is the inactivation of the mechanism for transporting solutes into the organisms. Support for this conclusion was from Quetsch and Danforth (1964),

and Beerens _et_ _al_. (1965), who all showed that at lower temperatures the uptakes of solutes was prevented. Enzymes denatured at low temperature have been reported by Shukuya and Schwert (1960), and Dua and Burris (1963). It is also now established that proteins and presumably therefore permeases, can be denatured at low temperatures (Brandts, 1967). Rose and Evison (1965) reported that the minimum temperatures for growth of mesophilic strains were approximately the same as those at which the organisms ceased to respire exogenous glucose and accumulate glucosamine. However, _Corynebacterium_ _xerosis_ continued to respire exogenous glucose and to accumulate glucosamine well below minimum temperature for growth, and the basis of the minimum temperature for growth of this organism may be due to the changes in the properties of the cell wall (Farrell and Rose, 1965).

More understanding about minimum temperature of growth of micro-organisms comes from Ingraham and his colleagues. Ingraham (1958) proposed that the marked increase in the $\mu$ value for growth rate of _E_. _coli_ at low temperature which resulted in the existence of a minimal temperature for growth, was a sequence of metabolic damage. A report from Ng _et_ _al_. (1962) supported this and showed that release from glucose repression of the induction of βeta-galactosidase occurred at a temperature coincident with low temperature damage. They suggested that a major reason for the existence of a minimum temperature of growth

is that regulation of the synthesis of enzymes is deranged at low temperature. Mutants which could be induced to form tryptophanase at low temperature have been isolated (Ng and Gartner, 1963). The constitutive synthesis of tryptophanase at low temperature requires at least two mutational events and the transcription of the structural gene might be directly affected by low temperature (Ingraham and Marr, 1965). However, mutants that were a single mutation and could lower the minimal temperature for growth under a specific environment have been isolated (Ingraham and Marr, 1965). The effect of temperature of growth on the synthesis of βeta-galactosidase was reported by Marr _et al_. (1964). Both a constitutive strain and a fully induced strain gave identical responses. The differential rate was constant above 20°C, but below that temperature the rate of enzyme synthesis decreased. Another type of mutant that cannot grow at low temperature has been isolated (O'Donovan _et al_., 1965). The so-called cold-sensitive mutants resulted from increased feed-back inhibition and affected allosteric proteins (O'Donovan and Ingraham, 1965). Recently, Condon and Ingraham (1967) reported a mutant of _Ps. putida_ with a cold-sensitive lession in the mandelate oxidation pathway. This group of workers emphasizes the control of enzyme biosynthesis as the main factor to determine the minimal temperature of growth.

A commonly cited theory of minimal temperature for growth is the lipid theory. This theory is based on the general observation that organisms grown at low temperature have a greater proportion of unsaturated fatty acids or of more highly unsaturated fatty acids in their lipids than if they are grown at low temperature (Gaughran, 1947). The temperature at which lipids become so highly unsaturated that they no longer fulfill their essential structural role in the cell is the minimal temperature for growth of that cell. However, reports from Marr and Ingraham (1962), Shaw and Ingraham (1965) concluded that the fatty acid composition of cells does not determine the minimal temperature of growth

## IV. THE BASIS OF MAXIMAL TEMPERATURE OF GROWTH

Several factors are thought to be involved in the maximum temperature for growth of micro-organisms, these include denaturation of enzymes, denaturation and possible degradation of DNA and RNA, and changes in the properties of membrane lipids.

Enzyme denaturation is usually assumed to be a major factor. Edwards and Rettger (1937) reported excellent agreement between the maximum temperature for growth of several bacilli and the temperatures at which certain respiration enzymes were inactivated. The inacti-

vation or reduction of respiratory and TCA cycle enzyme activities at higher temperature was also reported by Hagen and Rose (1962), Evison and Rose (1965), Upadhyay and Stokes (1963a, 1963b), Burton and Morita (1963), Langridge and Morita (1966), Purohit and Stokes (1967), Sinclair and Grant (1967), Mathemeier and Morita (1967). The loss of enzyme activity and possibly also inactivation of enzyme synthesis are major factors in determining maximal temperature of growth.

Denaturation and degradation of nucleic acids as factors in determining the maximum temperature for growth have been recently reported. Strange and Shon (1964) observed that conditions which accelerated the death rate of _Aerobacter aerogene_ at higher temperature also increased the rate of degradation of endogenous RNA. The thermal stability of RNA and ribosome in _B. stearothermophilus_ and _E. coli_ has been studied (Saunders and Campbell 1966). Sogin and Ordal (1967) demonstrated that the ribosomes and ribosomal RNA were almost completely broken down due to the heat treatment, however, these components could be regenerated during recovery process. Pace and Campbell (1966) found that there was correlation of denaturation temperature of ribosome and maximum growth temperature. In a further study, they reported the Guanine-Cytosine content of the ribosomal RNA preparation tended to increase with increasing growth

temperatures (Pace and Campbell, 1967). Similar results were obtained by Stenesh and Yang (1967), and Stenesh and Holozo (1967). The effect of temperature on the stability of DNA has been reported by Marmur and Doty (1959), and Marmur (1960). However, Marmur (1961) has shown that DNA lability was unrelated to maximum growth temperature.

Differences in lipid constitution have been suggested to be involved in the limitation of growth at high temperatures. Byrne and Chapman (1964) found that as the temperature increased, the fine structure of liquid-crystalline membrane lipid disappeared and the hydrocarbon moiety began to flex and twist until finally melting of the chain occurred. Luzzati and Husson (1962) reported that little variation in temperature might induce drastic changes in the liquid-crystalline regions and could play a role in the biological mechanisms where selective permeability was involved. The selective leakage of cellular components, especially RNA derivatives, into surrounding medium was found to occur at elevated temperatures, accompanied by the lysis of cells (Hagen *et al.*, 1964, Haight and Morita, 1966, Wong *et al.*, 1966, and Malcolm, 1967). Best (1967) reported that the autolysis of a psychrophile accompanied the release of soluble peptide at high temperatures. However, there is no direct evidence that this event is the primary cause of cell death. Stanley and Morita (1967)

recently showed that NaCl concentration affected maximal temperature for growth and might relate to the permeability of Vibrio marinus.

Single mutational alternation in the amino acid sequences of a protein helps in the understanding of maximum temperature for growth. Heat-sensitizing alternation of protein molecules of enzymes has been reported (Maas and Davis, 1952; Horowitz and Fling, 1956; Bock and Neidhardt, 1966). Regulation of enzyme synthesis could also be a major factor. Horuichi et al. (1961) have isolated a mutant of E. coli in which the regulation of βeta-galactosidase synthesis has become temperature-sensitive. Sadler and Novick (1965) presented information on a mutant in which the actual information of the aporepressor appeared to be temperature-sensitive. Gallant (1962), and Gallant and Stapleton (1963) have studied a mutant of E. coli in which the synthesis of an aporepressor for alkaline phosphatase has become temperature-sensitive.

Neidhardt and his associates have taken advantage of temperature-conditional lethal mutation (Epstein et al., 1963) to study the synthesis of RNA and its regulation. Eidlic and Neidhardt (1965a) reported that a temperature shift from 30°C to 37°C caused the depression of the two valine-controlled enzymes in the mutant studied. Certain aminoacyl-sRNA synthetases are temperature-sensitive

in the mutants that cannot grow at 37°C (Eidlic and Neidhardt, 1965b). A shift of temperature from 30 to 40°C resulted in a rapid discharging of valyl-sRNA co-ordinate with the cessation of protein synthesis (Bock et al., 1966). Temperature-sensitive DNA synthesizing enzyme also has been reported (Fangman, 1966; Kohiyama et al., 1966). Temperature sensitivity of macromolecular synthesizing enzymes could therefore be a major factor in determining maximal temperature of growth of certain micro-organisms.

## V. THE STUDIES OF MICROCOCCUS CRYOPHILUS

Psychrophiles are different from mesophiles in several aspects. It is obvious that by comparing the physiological and biochemical responses of psychrophiles and mesophiles to variation in temperature, the factors responsible for the differences might be elucidated. Some differences between these two groups of organisms have been found (Section II). However, the psychrophiles and mesophiles that have been compared have been different species that differed substantially in characteristics probably unrelated to their responses to growth temperature. Ideally, the compared micro-organisms in such studies should be identical except for their ability to grow at lower or higher temperature and except for these characteristics which

bear directly upon this ability. A mutant paired with its parent would fit the requirements.

*Micrococcus cryophilus* was first isolated by McLean *et al.* (1951) and was mainly used for cytologic studies because of its unusually large size (DeLamater and Woodburn, 1952 and Bisset, 1954). Apart from cytological importance, *Micrococcus cryophilus* possesses two other interesting characters, i.e. its ability to grow well at 0°C and its inability to grow above 24°C. Little is known about the phenomena.

Malcolm (1967) studied several physiological and biochemical characters of *M. cryophilus* with a view to increasing our knowledge and understanding of the biological basis for maximum growth temperature in this organism. The results and suggestions of Malcolm provide valuable information and guidelines for further study. The first aim of this present work is to isolate mesophilic mutants from *M. cryophilus*. A study of the general and basic characters of the mutants and the parent might help to elucidate the basis of the essential differences between psychrophiles and mesophiles, and also provide further understanding of the maximum growth temperature of *M. cryophilus*.

# MATERIALS AND METHODS

## I. GENERAL

### Organisms and Media

Obligately psychrophilic *Micrococcus cryophilus* ATCC 15174, later referred to as ATCC 15174, and its mesophilic mutants *Micrococcus cryophilus* ATCC 15174-T8 and ATCC 15174-M19, later referred to as T8 and M19 respectively, were used throughout the experiment.

The behaviour of the organisms was studied in complex medium, Trypticase Soy Broth (TSB)or Trypticase Soy Agar (TSA) (Baltimore Biological Labs.). Minimal medium in which glutamate served as sole carbon and nitrogen source was occasionally used. This contained $KH_2PO_4$, 5g; Sodium citrate, 5g; NaCl, 5g; $MgSO_4.7H_2O$, 0.08g; $MnCl_2.4H_2O$, 0.01g; $FeCl_3$, 0.001g; monosodium glutamate, 7.5g and distilled water to 1 liter. The pH was adjusted to 7.2 with 1N KOH (Malcolm, 1967). All media were sterilized at 121°C for 15 minutes. Stock cultures were maintained on TSA at 0°C.

### Growth Conditions

Cultures were grown in TSB medium in a New Brunswick

Psycrotherm incubator shaker (New Brunswick Scientific Co., New Jersey). Unless stated otherwise, the cultures were grown in 300 ml flasks containing 100 ml medium and shaken at 300 r.p.m.

## Preparation of Resting Cells

Exponentially growing cells were harvested by centrifugation at 25,000 x g for 10 minutes in a refrigerated centrifuge (Sorvall RC2-B) at 0°C and washed twice with potassium phosphate buffer (0.05M, pH 7.2). The cultures were re-suspended and adjusted to the desired concentration using a Spectronic 20 spectrophotometer (Bausch & Lomb).

## General Characteristics of the Organisms

The growth range was tested by streaking the cultures on TSA plates. Growth was recorded after incubation at 37, 34 and 30°C for 3 days, 20 and 14°C for 5 days, 8, 4 and 0°C for 2 weeks.

Gelatin hydrolysis (Smith's method), nitrate reduction, relation to free oxygen, starch hydrolysis, relation to litmus milk, rennet production, Indol Test (Kovac's method) and hydrogen sulphide production were determined according to the procedures suggested in

"Manual of Microbiological Methods" (1957). The utilization of citrate was tested by the method of Simmon (1926), urease by that of Ferguson and Hook (1943), methyl-red and Voges-Proskauer (MR-VP) reaction by that of "Standard Methods for the Examination of Dairy Products" (1960), using Redi-Disc (Pen. Biol. Lab.). The presence of catalase was detected by the addition of 3% $H_2O_2$, oxidase with 1% para-aminodimethyl aniline monohydrochloride (Eastman Kodak Co.) (Gordon and Mcleod, 1928). Extracellular nucleases were tested by a method based on Jeffries *et al*. (1957). Resistance to high salt concentration was assayed by streaking cultures on mannitol salt agar (Difco), penicillin sensitivity on TSA plates containing 100 I.U. penicillin G (Difco), resistance to sodium lauryl sulphate and lysozyme by immersing sterile filter paper discs (Difco) in 5% sodium lauryl sulphate and 50$\mu$g/ml lysozyme (Difco) respectively and placing on the surface of a TSA plate previously poured with test organisms. Sensitivity to these materials was observed by the formation of an inhibition zone. All tests were carried out at 20°C. Carbohydrate fermentation was tested with the method of Sanders *et al*. (1957), using both Difco-discs and Redi-discs (Pen. Biol. Lab.) in the presence of phenol red as indicator. Results were read after 6 and 18 hours of incubation at 30°C for mutants, and 20°C for the wild type. Fermentation of carbohydrate

was shown by acid formation within 18 hours and its presence indicated by a colour change of the medium to yellow. In all cases controls, without culture, were included.

## Thermal Death Point and Thermal Survival Curve

The thermal death point (TDP) of organisms was measured by adding washed cells at a certain optical density (OD) into tubes containing 10 ml melted TSA medium. The tubes were held at different temperatures for 10 minutes. Thermal survival curves were determined by withdrawing a series of tubes containing 10 ml liquid broth at given time intervals and plating out with suitable dilutions. Timing was started when the control tube, containing a thermometer, reached the desired temperature. Triplicates were used in each measurement.

## Selection of Mutants with Increased Growth Temperatures

The wild strain, ATCC 15174, was grown exponentially at 14°C in the minimal medium. Five ml samples of culture were placed in sterilized petri dishes and irradiated with a 30 watt UV-lamp (General Electric Co.) from 10 seconds to 10 minutes, following which 0.1 ml

of the irradiated culture were surface plated on TSA. After incubation at 30°C for 3 days the phenotypic colonies were replicated and examined under the microscope to check for purity of culture. Only those colonies that retained the ability to grow at 30°C and were morphologically similar to the wild strain were considered to be mutants.

### Preparation of Biologically Active DNA

The method was based on that of Marmur (1961). Exponentially growing cells were harvested by centrifugation in a refrigerated centrifuge (Sorvall RC2-B) at 0°C and washed once with 40 ml saline-EDTA (0.15M NaCl, 0.1M disodium-EDTA, pH 8.0). After collecting about 3g of wet packed cells they were re-suspended in a total volume of 25 ml saline-solution (0.15M NaCl, pH 8.0). Lysis was carried out by the addition of 2.0 ml of 20% sodium lauryl sulphate and the mixture placed in a 60°C waterbath for 10 minutes. Sodium perchlorate was added to the mixture to a concentration of 1M. After thorough mixing, deproteinization was done by adding an equal volume of chloroform-isoamyl alcohol reagent (24:1, v/v) and the mixture rapidly shaken for 15 minutes. After centrifugation at 0°C for 5 minutes at 6,870 x g, the clear supernatant was removed by pipette.

Then, two volumes of 95% ethanol were carefully layered over it. The collected threads of nucleic acids were pressed dry on the side of the beaker and dissolved in 15 ml saline-citrate (0.15M NaCl - 0.015M sodium citrate). Three successive deproteinizations were carried out following which the nucleic acids were dissolved in 0.5 supernatant volumes of saline citrate. Then 0.3 ml of 0.5% (w/v) ribonuclease (RNase) solution was added. The resultant mixture was incubated at 37°C for 30 minutes to digest the contaminating RNA. Deproteinization was again performed and the deoxyribonucleic acid (DNA) collected by winding. After dissolving the DNA in 9.0 ml saline citrate, 1.0 ml acetate-EDTA (3.0M sodium acetate - 0.001M EDTA, pH 7.0) was added, and while stirring rapidly, 0.60 volumes of isopropanol were added drop by drop. The threaded DNA was collected by centrifugation for 15 minutes at 24,000 x g at 0°C. The purified DNA was dissolved in 10.0 ml saline-citrate which yielded a clear solution. About 1 - 2 mg of DNA was obtained. This preparation was stored at 2°C and was used for transformation and denaturation temperature studies.

## Transformation of Growth Temperature Character

Transformation procedures were according to those of Fox and Hotchkiss (1957) except all were carried out in a 20°C Environ-room

(Lab-Line Inst. Corp.). Wild type ATCC 15174 grown exponentially on TSB medium was frozen with 10% glycerol and kept at about -20°C for 18 hours to maintain a competent recipient cell population. The culture was thawed and diluted 1:10 in 25 ml TSB medium containing 0.02g bovine serum albumin (CalBiochem.) and $10^{-3}$ - $10^{-4}$ M $Ca^{++}$ ion. T8 or M19 DNA preparation containing about 0.4 mg DNA was added. The mixture was shaken on an Eberback shaker (Eberback Inc., Ann Arbor) at about 50 r.p.m. At different time intervals, 1 ml of culture was removed into a 15 x 150 mm test tube containing 0.1 ml of 50 $\mu$g/ml DNase (CalBiochem) in 0.03M $MgSO_4$ (pH 7.0). After incubation at 20°C for 2 - 4 hours to permit the expression of the transforming property, 0.1 ml cultures were surface plated on TSA. The colonies formed could be observed after incubation at 30°C for 3 days. In no case did the controls of streaks of ATCC 15174 or DNA preparations of mutants give rise to any colony. The plates forming colonies were replicated on TSA and minimal medium, and incubated at 30°C and 20°C. DNase and Albumin solutions were sterilized by filtration through Millipore membrane filter paper of 0.3 micron pore size.

## Determination of DNA Melting Temperature (Tm)

Biologically active DNA preparation (Marmur, 1961) was

used in this study. The method was that of Marmur and Doty (1962). The OD of the preparation was adjusted with saline-citrate (0.15M NaCl - 0.015M sodium citrate, pH 7.0) to about 0.4 at 260 mμ. The DNA was contained in a 3 ml-glass stoppered quartz cuvette having a 1 cm light path. The samples were placed in a Beckman DU spectrophotometer chamber, the temperature of which could be raised by circulating hot water through two thermal spacers (Beckman) on either side. To protect the photocell, other spacers were slowly circulated with water at 20°C. The Tm values were obtained by reading the OD of samples immediately after equilibrating 10 minutes at the desired temperature. A sharp increase in the absorbance occurred in the transition range during which the DNA was denatured. The optical density at each temperature, corrected for the thermal expansion, was divided by the values at 25°C and the ratio (relative absorbance) plotted versus the temperature of the solution. The temperature corresponding to half the increase in the relative absorbance was designated as the Tm.

The Guanine-Cytosine (G-C) content of DNA was calculated by the equation $Tm = 69.3 + 0.41\ (G\text{-}C)$ (Marmur and Doty, 1962).

## II. GROWTH, RESPIRATION AND INTERMEDIARY METABOLISM

### Measurement of Growth

1. Viable cell counts: Dilutions were made in 0.1% peptone water at 0 - 4°C. Ten 0.01 ml samples of a suitable dilution were plated on to the surface of a plate poured 24 hours previously and allowed to dry at 20°C. Surface plates were used to avoid possible heat damage to the cells by mixing with melted agar and to distinguish from possible contamination. An incubation time of 96 hours at 20°C was sufficient to allow colonial numbers to be counted easily. This procedure was used in all viability studies.

2. Turbidity: Optical density of cultures at 600 mμ was compared with a blank of unincubated medium in a Bausch & Lomb Spectronic 20. When it was necessary to read higher densities, appropriate corrections were made from known dilutions of the more dense sample.

3. Dry weight: Dry weight of cultures was measured using a standard curve of known dry weight versus optical density of the suspensions (Fig. 1). Early midlogarithmic growing culture was harvested, washed twice at 0°C and re-suspended in the medium used. OD of

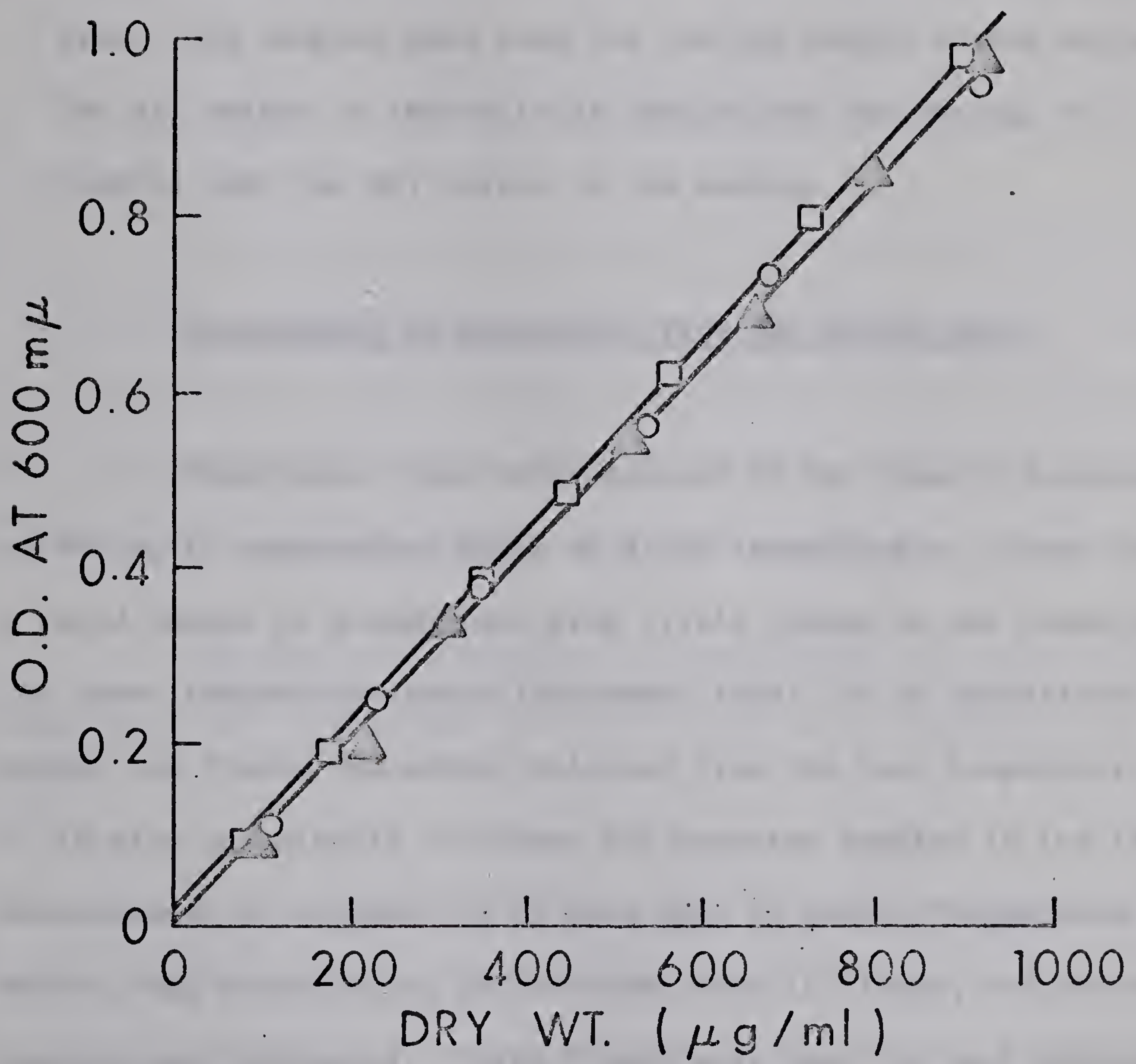

Fig. 1 Calibration curves relating turbidity to dry weight of cells

○——○ ATCC 15174
▲——▲ T8
□——□ M19

5 ml samples of each appropriate dilution were read at 600 mμ and transferred to a dried aluminum-foil cup of known weight and the whole unit was immediately placed in a drying oven at 105°C for 18 hours. No noticeable growth was found during the procedures. Six samples were used for the dry weight of the medium. The dry weight of the cells at each OD was the average of 3 samples less the dry weight of the medium.

## Measurement of Generation Time and Growth Rate

Generation times were measured by the time of doubling OD at 600 mμ in exponential phase at given temperatures. Since there is a rapid change of growth rate with little change in the temperature in the lower temperature range (Ingraham, 1958), it is undesirable to remove the flasks containing cultures from the test temperature and it is also undesirable to return the measured samples to the flasks. Measurements of increase in OD were made by mixing the inoculum and TSB medium, and distributing the mixtures into 12 flasks, and the measured samples were discarded. Three flasks were used for each measurement, each flask containing 50 ml of TSB medium. In all cases the measurements were in the exponential phase of cell growth and the turbidity was linearly proportional to incubation time.

The growth rate, k, in $hr^{-1}$, was calculated from the equation:

$$k = \frac{\ln 2}{\text{generation time}}$$

## Respiratory Studies

The respiratory activities of the organisms at different temperatures were determined as described by Umbreit *et al*. (1964) with the constant volume Warburg respirometer (Precision Scientific Co., Chicago) fitted with cooling coils through which water was circulated from a low temperature waterbath (Blue M Electric Co.).

All physical constants for determining the flask constant under different conditions are from "Handbook of Chemistry and Physics" (1963).

Resting cells were re-suspended in 0.1M phosphate buffer, pH 7.2 and adjusted to a cell concentration suitable for use in the Warburg respirometer. The centre well of each Warburg flask contained 0.2 ml 20% (w/v) KOH to adsorb released $CO_2$. A portion of suspension containing 3 mg to 10 mg dry weight of cells, usually 5 mg, was added to the flask and the total volume adjusted to 2.5 ml with buffer.

The side arm contained 0.5 ml of solution of an oxidizable substrate (3%, w/v, pH 7.2), or 0.5 ml water when measuring the respiration of endogenous reserves. After the Warburg flasks had been attached to the manometers, they were equilibrated in the waterbath for 10 minutes and then the manometer tap was closed, before the substrate was tipped from the side arm. The uptake of oxygen by the suspension was followed over a period of at least 2 hours. The barometer contained all substrates except cell suspension.

The respiratory activities were calculated as the $QO_2$ values ($\mu$l oxygen consumed/mg dry weight/hr) or oxygen uptake ($\mu$l oxygen consumed/mg dry weight).

## The Effect of Chloramphenicol on Respiration

The effect of protein synthesis on respiratory activity was determined by the use of chloramphenicol (Parke-Davis & Co.). Each Warburg reaction flask contained about 5 mg dry weight of cells and 0.1M $K_2HPO_4$ buffer, pH 7.2 to a total volume of 2.0 ml. The first side arm of each flask was charged with 0.5 ml oxidizable substrate (3%, w/v) and the substrate was tipped in after the manometer had been equilibrated for 10 minutes. 0.5 ml of chloramphenicol (360 $\mu$g/ml) which had been charged into the second side arm of the flask, was tipped

into the main cell at the desired time. The central well contained 0.2 ml 20% KOH. The respiratory activity was expressed as described previously.

## Determination of Protein

The protein content of samples was estimated either by the Folin method of Lowry et al. (1951) when the samples contained a high concentration of nucleic acids, or by the optical method of Warburg and Christian (1941) when the samples contained only small amounts of nucleic acid.

The Folin method of Lowry et al. (1951) utilizing Folin-Ciocalteau phenol reagent was as follows:

A suitable 0.5 ml dilution of sample was mixed with 5 ml of a mixture of 1 part of 0.5% copper sulphate in 1% sodium tartarate, to 50 parts of 2% $Na_2CO_3$ in 0.1 N NaOH and then incubated at 37°C for 30 minutes. After cooling to room temperature, 0.5 ml of Folin-Ciocalteau reagent (1:1, aqueous) was added and the mixture kept for 20 minutes at room temperature. The intensity of the blue colour was read at 500 m$\mu$ with a Spectronic 20. A standard curve was prepared using bovine serum albumin (CalBiochem) (Fig. 2).

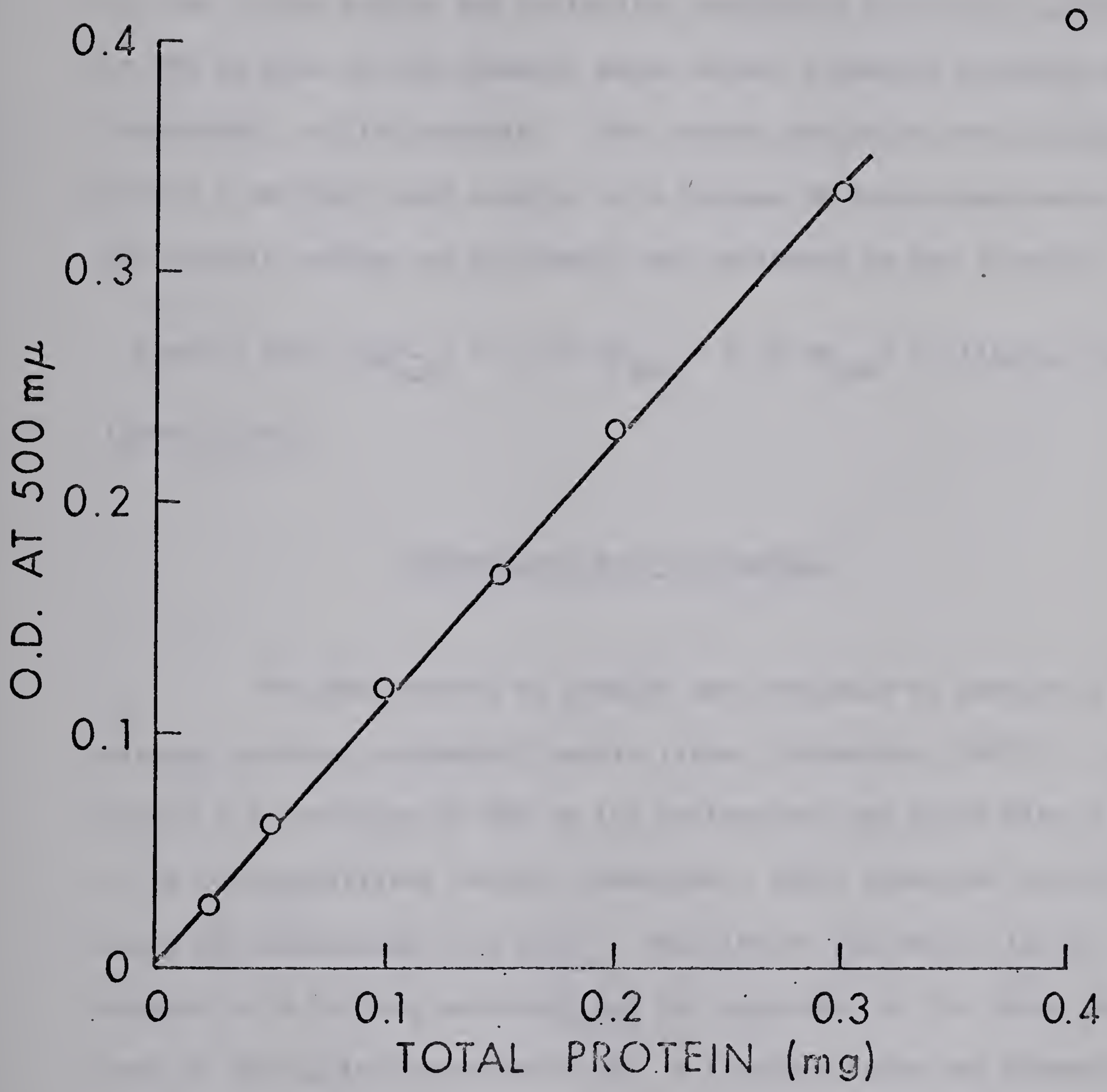

Fig. 2 Standard curve for concentration of protein

The optical method of Warburg and Christian (1941) depends upon the relative optical densities of the protein solution at 260 mμ (due to the purine and pyrimidine components of nucleic acids) and at 280 mμ (due to the aromatic amino acids, primarily tyrosine and tryptophan, of the protein). The optical densities were measured with a 1 cm-light path cuvette in a Beckman DU spectrophotometer. The protein content of the sample was estimated by the formula:

$$\text{protein conc.}(\text{mg}/_{\text{ml}}) = [1.55\ \text{OD}_{280} - 0.76\ \text{OD}_{260}] \times \text{dilution factor}$$

(Layne, 1957).

## Ribonucleic Acid Estimation

The RNA content of samples was estimated by measuring the orcinol reacting compounds, mainly ribose (Schneider, 1957). A suitable 2.0 ml dilution of RNA or its derivatives was mixed with 2.0 ml of 1% re-crystallized orcinol (Schneider, 1957) dissolved in 100 ml conc. HCl containing 0.5g $FeCl_3$. The mixture was heated for 20 minutes in a boiling waterbath and the intensity of the green colour read at 660 mμ in a Spectronic 20. A standard curve was prepared using yeast soluble RNA (CalBiochem). (Fig. 3).

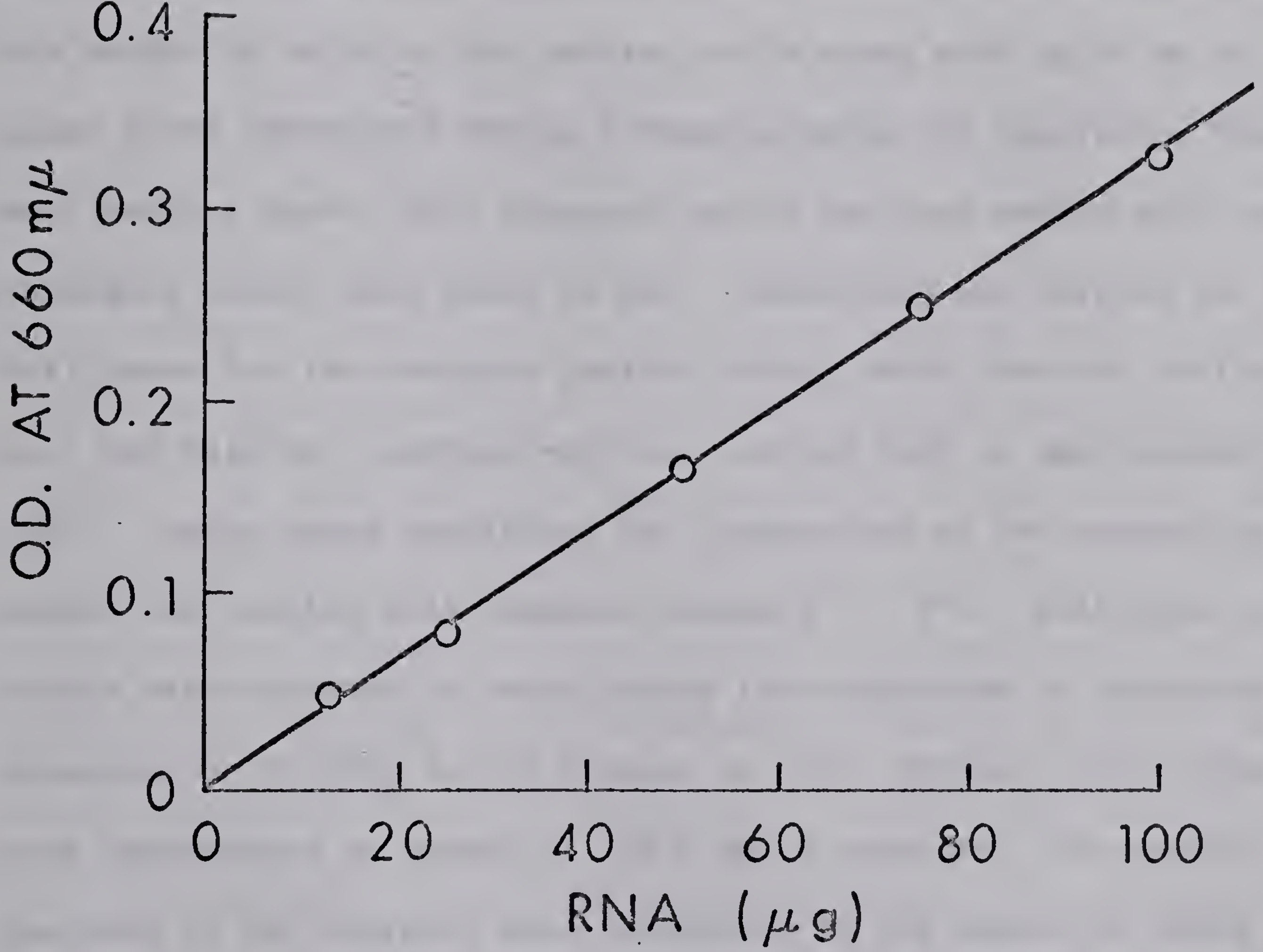

Fig. 3 Standard curve for concentration of RNA

## Preparation of Cell-free Extracts

Crude cell-extracts of organisms for use in the measurement of enzyme activities were prepared by sonic oscillation in a Branson Sonifier, model S-75, fitted with a Rosett cooling cell. Cells were harvested and washed twice with 0.05 M phosphate buffer, pH 7.5, at 0°C and suspended to a final concentration of 20 mg/ml dry weight of cells in the cooling cells along with 1g:10 ml of glass beads (Minnesota Mining & Manufacturing Co: Superbrite Pavement Marking Beads, 200$\mu$ diameter) which had been washed with concentrated nitric acid prior to use. Sonication was carried out at full power for two 4-minute periods during which time the cooling cell was held in a methanol-dry ice cooling bath at approximately -10°C. Under these conditions the temperature of the suspension within the cooling cell remained around 0°C - 2°C. Cell-free extracts were obtained by centrifuging the suspension of disrupted organisms at 25,000g for 20 minutes at 0°C. Extracts were either used immediately or stored at -20°C until required. The protein contents of the extracts were determined by the method of Lowry et al. (1951).

## Assay of Enzymes of Intermediary Metabolism

The activities of all the enzymes studied were calculated from initial velocities determined over a period during which the amount of substrate consumed against time was linear. All activities were calculated as specific activities ($\mu$M substrate consumed/mg protein/hr).

The activities of citrate synthetase, aconitate hydratase, isocitrate dehydrogenase, fumerate hydratase, L-malate dehydrogenase, isocitratase, malate synthetase, glutamic dehydrogenase and glutamic-oxaloacetic transaminase were measured spectrophotometrically in a Beckman DU Spectrophotometer fitted with constant temperature cuvette spacers. The reactions were carried out in 1 cm quartz cuvettes and unless otherwise stated, all reaction constituents except the cell extract were equilibrated in the cuvettes at the test temperature before starting the reaction. The biochemicals used were obtained from either Sigma Chem. Corp. or CalBiochem. Citrate synthetase (citrate oxaloacetate-lyase (Co A-acetylating); E.C.4.1.3.7.) activity in cell extracts was determined by the method of Dixon and Kornberg (1959). This involved measuring the rate of decrease of optical density at 232 m$\mu$ consequent upon breakage of the thio-ester bond of acetyl co-

enzyme A in the presence of oxaloacetate (Stadtman, 1957). Each cuvette contained, in 2.1 ml, tris buffer (90 $\mu$M; pH 7.1) and acetyl co-enzyme A (0.125 $\mu$M; pH 7.1). One-tenth ml of cell extract containing approximately 100 $\mu$g protein was added to the test cell and the change in extinction at 232 m$\mu$ recorded for several minutes to detect the possible presence of acetyl coenzyme A deacylase. Cis-oxalacetate (5 $\mu$M) was then added; the initial rate of the rapid decrease in optical density is proportional to the citrate synthase present. The specific activities were calculated from the change in extinction at 232 m$\mu$ for the thioester bond of acetyl coenzyme A which is $4.5 \times 10^3$ (Stadtman, 1957).

Aconitate hydratase (citrate (isocitrate) hydrolyase; E.C.4.2.1.3.) was assayed by a method based on that of Racker (1950) which depends upon measuring the increase in extinction at 240 m$\mu$ attendant upon the conversion of citrate to Cis-aconitate. Each cuvette was charged with sodium citrate (87 $\mu$M; pH 7.4) and sodium phosphate buffer (145 $\mu$M; pH 7.4) in 2.9 ml water. The reaction was started by adding 0.1 ml of a portion of cell extract containing approximately 100 $\mu$g protein; 0.1 ml water was added to the control cuvette. The increase in extinction at 240 m$\mu$ caused by the formation of Cis-aconitate, was followed at 30 second intervals for a period of

5 minutes. Specific activities were calculated using the value for the extinction in 3.0 ml water of 1 μM Cis-aconitate at 240 mμ quoted by Williams and Rainbow (1964).

Isocitrate dehyrogenase ($NADP^+$-linked) was assayed by following at 340 mμ, the increase in extinction on reduction of $NADP^+$ (Kornberg and Pricer, 1951). Each cuvette contained sodium DL-isocitrate (0.5 μM; pH 7.0), potassium phosphate buffer (100 μM, pH 7.0), $NADP^+$ (0.5 μM), $MgCl_2$ (10 μM) in 2.9 ml water. The reaction was started by adding 0.1 ml of cell extract containing about 100 μg protein, and the increase in extinction at 340 mμ was followed at 30 second intervals over a period of 3 minutes, with a blank reaction mixture lacking cell extract. The extinction of a second blank reaction mixture containing all of the constituents except DL-isocitrate was measured at the beginning and at the end of the period of observation. Specific activities were calculated from the change in extinction using the molar extinction coefficient for $NADPH.H^+$ quoted by Horecker and Kornberg (1948).

Fumarate hydratase (L-malate hydrolyase; E.C.4.2.1.2.) activity was assayed using a method based on that of Racker (1950) which depends on measuring the decrease in the extinction at 300 mμ attendant on the conversion of fumerate to L-malate. Each cuvette

contained sodium fumarate (49 μM; pH 7.3) and sodium phosphate buffer (95 μM; pH 7.3) in 2.9 ml water. The reaction was started by adding 0.1 ml cell extract containing about 400 μg protein; 0.1 ml water was added to the control reaction mixture. The decrease in extinction at 300 mμ, caused by the decrease in the concentration of fumarate, was followed at 30 second intervals over a period of 5 minutes. Specific activities were calculated using a value for the extinction of μM fumarate in 3.0 ml mμ of 0.015.

L-malic dehydrogenase ($NAD^+$-linked) activity was assayed using oxaloacetate as substrate and following the decrease in extinction caused by the oxidation of $NADH.H^+$ (Ochoa, 1955). Each cuvette contained sodium phosphate buffer (150 μM; pH 7.4), potassium oxalocetate (0.76 μM; pH 7.4) and $NADH.H^+$ (0.15 μM; pH 7.4) in 2.9 ml water. The reaction was started by adding 0.1 ml of cell extract containing about 100 μg protein and the decrease in extinction at 340 mμ was followed at 30 second intervals over a period of 3 minutes. Specific activities were calculated using the molar extinction coefficient for $NADH.H^+$ quoted by Horecker and Kornberg (1948).

*Iso*-citratase ($L_s$-isocitrate glyoxylate-lase; E.C.4.1.3.1.) was assayed by measuring the rate of increase of optical density at 324 mμ consequent upon the formation of glyoxylic acid phenylhydrazone

from glyoxylate produced by the action of this enzyme from iso-citrate, and phenylhydrazine (Dixon and Kornberg, 1959). Each cuvette contained sodium phosphate buffer (150 $\mu$M; pH 6.9), $MgCl_2$ (15 $\mu$M; pH 6.9), phenylhydrazine HCl (10 $\mu$M; pH 6.9), cysteine HCl (6 $\mu$M; pH 6.9) in 2.9 ml water. The reaction proceeded on addition of 5 $\mu$M potassium iso-citrate and after a lag of approximately 1 minute, the change in extinction at 324 m$\mu$ was linear for about 4 minutes, the rate being proportional to the enzyme concentration. Specific activities were calculated using the molar extinction coefficient for glyoxylic acid phenylhydrazine of $1.7 \times 10^4$ at 324 m$\mu$.

Malate synthetase (L-malate glyoxylate lyase (CoA-acetylating): E.C.4.1.3.2.) activity was assayed by measuring the rate of decrease of optical density at 232 m$\mu$ consequent upon breakage of the thio-ester bond of acetyl CoA (Stadtman, 1957) in the presence of glyoxylate (Dixon and Kornberg, 1959). The reaction system contained in 2.9 ml, tris buffer (90 $\mu$M; pH 7.1), $MgCl_2$ (10 $\mu$M; pH 7.1), acetyl coenzyme A (0.125 $\mu$M; pH 7.1), and 0.1 ml of cell extract containing approximately 100 $\mu$g protein. The change in optical density at 232 m$\mu$ was recorded for several minutes to detect the possible presence of an acetyl coenzyme A deacylase and then 5 $\mu$M sodium glyoxylate were added. The quantity of malate formed in the reaction mixture

corresponds to the amount of acetyl CoA utilized and the specific activities were calculated using a molar extinction coefficient at 232 mμ of $4.5 \times 10^3$ for the cleavage of the thio-ester bond of acetyl coenzyme A (Stadtman, 1957).

Glutamic dehydrogenase ($NAD^+$- or $NADP^+$-linked) activities were assayed in two ways by measuring either the decrease in optical density at 340 mμ as the reduced coenzyme is oxidized, in the case of the synthetic direction (Vender, _et_ _al_. 1965), or by the increase in extinction attendant upon the formation of reduced coenzyme as glutamate is oxidized. The synthetic reaction system contained tris buffer (90 μM; pH 7.5), $NH_4Cl$ (40 μM; pH 7.5), alpha-ketoglutaric acid (6.7 μM; pH 7.5), $NADPH.H^+$ or $NADH.H^+$ (0.15 μM; pH 7.5) in 2.9 ml. The reaction was initiated by the addition of 0.1 cell extract containing about 100 μg protein and the change in optical density at 340 mμ was followed at 30 second intervals over a period of 4 minutes. The decrease in OD when $NH_4Cl$ or cell extract was omitted was subtracted in each case to obtain the enzyme activity. Specific activities were calculated using the molar extinction coefficient for $NADH.H^+$ quoted by Horecker and Kornberg (1948). The oxidation of glutamate in the presence of oxidized coenzyme was measured according to Strecker (1955). Each cuvette was charged with phosphate buffer

(150 $\mu$M; pH 7.6), NAD$^+$ or NADP$^+$ (0.1 $\mu$M; pH 7.6) potassium glutamate (100 $\mu$M; pH 7.6) in 2.9 ml water and 0.1 ml cell extract containing approximately 100 $\mu$g protein. The increase in absorption at 340 m$\mu$ was recorded at 30 second intervals.

Glutamic-oxaloacetic transaminase (1-aspartate: 2-oxoglutarate aminotransferase; E.C.2.6.11.) activity was assayed by measuring the increase in OD at 280 m$\mu$ due to the formation of oxalacetate (Gunsalus and Stamer, 1955). Each cuvette contained sodium phosphate buffer (150 $\mu$M; pH 7.3), L-aspartate (30 $\mu$M), pyridoxal phosphate (10 $\mu$g), alpha-ketoglutarate (30 $\mu$M; pH 7.3), approximately 100 $\mu$g protein of cell extract and water to 3.0 ml. The reaction was started by addition of alpha-ketoglutarate at zero time. The increase of OD at 280 m$\mu$ was recorded at 30 second intervals for 5 minutes. Specific activity was calculated from the amount of oxalacetate formed.

The activities of pyruvate, alpha-ketoglutarate and succinate dehydrogenases, along with glutamic decarboxylase, were assayed manometrically by the conventional constant volume respirometer technique (Umbreit et al., 1964). Pyruvate dehydrogenase activity was assayed by measuring carbon dioxide evolution with sodium pyruvate as substrate and potassium ferricyanide as electron acceptor (Jaganathan and Schweet, 1952). Each flask was charged with

sodium pyruvate (100 $\mu$M), sodium bicarbonate (50 $\mu$M), $MgCl_2$ (20 $\mu$M) and thiamine pryophosphate (200 $\mu$g; adjusted to pH 6.0 just before use). These constituents were added as a solution which had been flushed with carbon dioxide gas for 2 to 3 minutes. A portion of cell extract containing approximately 3 mg protein and water to a volume of 3.2 ml were also added to the flask, the side arm of which contained potassium ferricyanide (100 $\mu$M). The flasks were attached to the manometers, and flushed with carbon dioxide gas for 10 minutes, with a glass manifold to ensure even gassing. The manometer units were then quickly transferred to the waterbath and equilibrated for 10 minutes. All measurements of $CO_2$ evolution by other enzymes followed the same procedures. After the potassium ferricyanide had been tipped in from the side arm, the evolution of carbon dioxide was followed during a period of 10 minutes. Control flasks contained all of the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

Alpha-ketoglutarate dehydrogenase activity in cell extracts was assayed by measuring carbon dioxide evolution in a system which contained alpha-ketoglutarate as a substrate and potassium ferricyanide as electron acceptor (Sanadi _et_ _al_., 1952). The

main compartment of each Warburg flask was charged with alpha-ketoglutarate (20 μM). The solution in the Warburg flasks was supplemented with bovine plasma albumin (30 mg), a portion of cell extract containing approximately 5 mg protein, and water to a volume of 3.2 ml. The side arm of the flask contained potassium ferricyanide (50 μM). After the potassium ferricyanide had been added from the side arm, the evolution of carbon dioxide was recorded during 10 minutes. Control flasks contained all the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

Succinic dehydrogenase activity in cell extracts was assayed by measuring the amount of oxygen uptake with succinate as a substrate in the presence of phenazine methosulphate as an electron carrier (Bernath and Singer, 1962). Each Warburg flask was charged with sodium phosphate buffer (150 μM; pH 7.6), KCN (30 μM; pH 7.6), cell extract containing approximately 3 mg of protein and water to 3.2 ml. Sodium succinate (60 μM; pH 7.6) and phenazine methosulphate (0.2, 0.1, 0.07, 0.05 or 0.04 ml of a 1%, w/v, solution) were added to the side arm. KCN was added last, the flasks immediately attached to the manometers and the stopcocks closed. The excess pressure was released momentarily after the units had been placed

in the Warburg bath. After equilibrating for 10 minutes the contents of the side arm were tipped into the flasks and the uptake of oxygen was recorded during 20 minutes. Control flasks contained all of the constituents of the reaction mixture except cell extract. The reciprocal of the $QO_2$ value (calculated over a period of 2 to 12 minutes) was then plotted against the reciprocal of the concentration of phenazine methosulphate and the oxygen uptake extrapolated to infinite phenazine methosulphate concentration. This value for the oxygen uptake was used to estimate the specific activities of succinic dehydrogenase in the extracts (Bernath and Singer, 1962).

Glutamic decarboxylase (L-glutamate 1-carboxy-lyase; E.C.4.1.1.15) activity was assayed by measuring carbon dioxide evolution in a system which contained L-glumatic acid as the substrate and pyridoxal phosphate as the co-factor (Vender _et_ _al_., 1965). The main compartment of each Warburg flask was charged with pyridoxal phosphate (0.30 mg) and acetate buffer (180 $\mu$M; pH 4.8) in the form of a solution that had been flushed with a carbon dioxide gas for 2 to 3 minutes. The solution in the flask was supplemented with cell extract, containing approximately 3 mg protein, bringing the total volume up to 3.2 ml. The side arm of the flask contained L-glutamic acid (45 $\mu$M). After the L-glutamate had been tipped in from the side

arm, the evolution of carbon dioxide was recorded during 10 minutes. Control flasks contained all the constituents of the reaction mixture except cell extract. Specific activities were calculated from the amount of carbon dioxide evolved.

## III. MACROMOLECULAR SYNTHESIS AND BREAKDOWN

### Assay of Amino acyl-transfer-RNA Synthetases

Amino acyl-t-RNA synthetases catalyze a reaction between ATP and various specific amino acids leading to the activation of the carboxyl group of the amino acid and the elimination of phosphate or pyrophosphate from ATP.

Two methods were used to assay for amino acyl-t-RNA synthetase activities. To lessen the problem of endogenous amino acid saturation of the enzymes, the cell extracts were dialyzed overnight against 0.006 M tris buffer (pH 7.5) plus 0.006 M mercaptoethanol at 1°C.

#### 1. Amino Acid Hydroxamate Formation

This method is based on the reaction

$$RCH(NH_2)COOX + NH_2OH \rightarrow RCH(NH_2)CONHOH + HOX$$

in which activated amino acids react with $NH_2OH$ under relatively mild conditions to yield the amino-acyl hydroxamate that is measured colorimetrically with $FeCl_3$.

The assay was similar to that reported by Stulberg and Novelli (1962). The reaction mixture contained, in 1 ml, Tris buffer (200 $\mu$M; pH 7.3), ATP (10 $\mu$M), amino acids (10 $\mu$M), salt-free $NH_2OH$ (1000 $\mu$M) and either cell extract containing approximately 100 $\mu$g of protein or denatured cell extract heated in boiling water for 30 minutes. Incubation proceeded for one hour and then the reaction was halted by the addition of 3 ml of colour assay reagent (10% $FeCl_3$, 10% TCA and 5% HCl). The precipitated protein was removed by centrifugation and the colour density at 540 m$\mu$ was read in a Spectronic 20 and compared with a standard curve obtained by using synthetic amino acid hydroxymate (Sigma) in the above procedures in the absence of enzyme (Fig. 4). Salt-free hydroxylamine was prepared by the method of Beinert _et_ _al_. (1953). Specific activity was calculated as $\mu$M hydroxamate formed/mg protein/hr.

2. Amino Acid Dependent Pyrophosphate Exchange

The method is based on the exchange reaction

$$RCH(NH_2)COOH + ATP \rightleftarrows AMP\text{-}OOC\text{-}CH(NH_2)R + PP$$

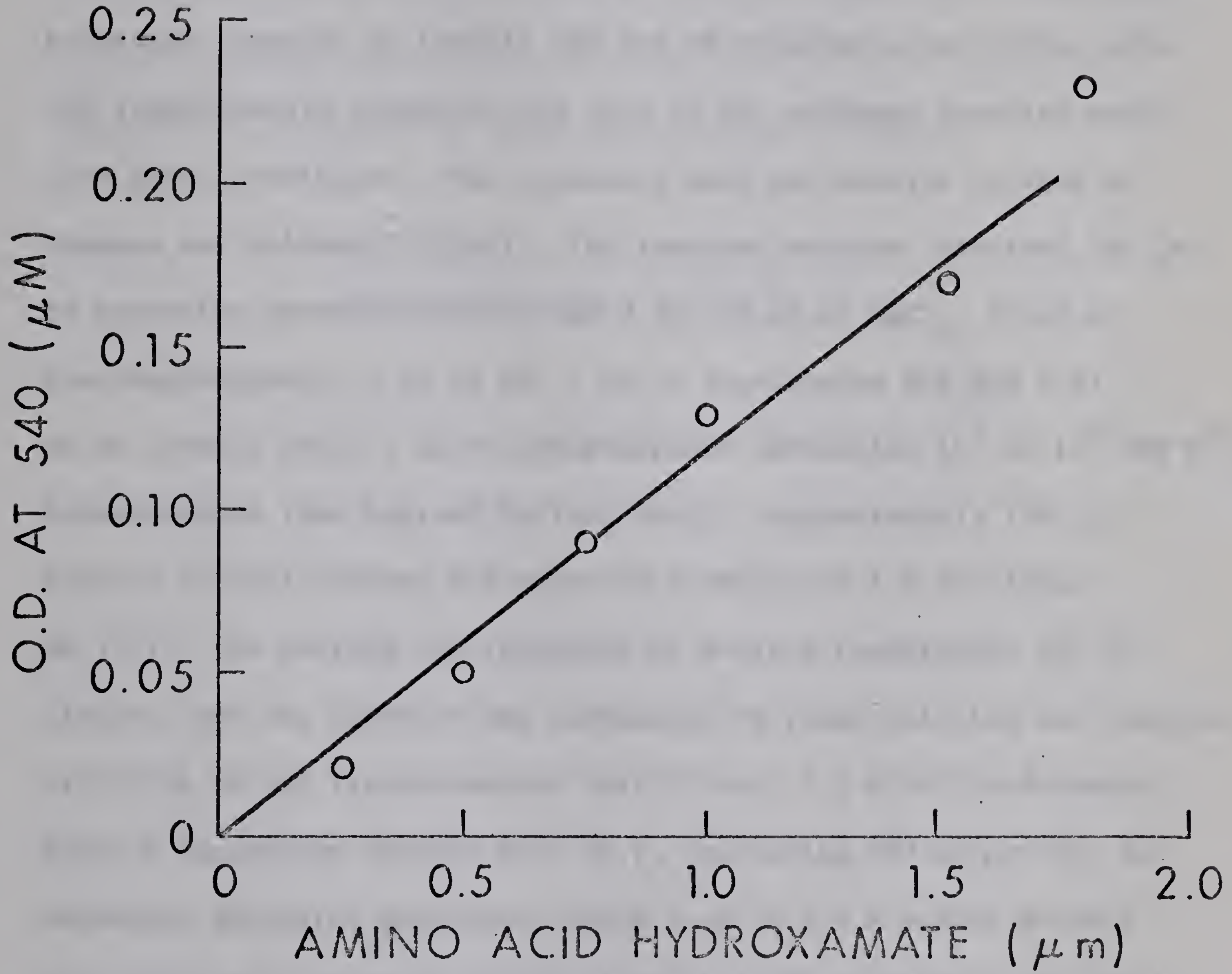

Fig. 4 Standard curve for concentration of phenylalanine hydroxamate

in which an amino acid-dependent exchange between ATP and pyrophosphate is observed. Thus, P-$P^{32}$ can be observed to exchange with the pyrophosphoryl moiety of ATP. The assay is quantitative only under conditions where the concentration of reactants remains constant throughout the time course of the reaction. Addition of sufficient potassium fluoride to inhibit ATP and PP hydrolytic activities without significantly affecting the rate of the exchange reaction satisfied these conditions. The procedure used was similar to that of Fangman and Neidhardt (1964). The reaction mixture contained 100 $\mu$M of potassium phosphate buffer (pH 7.3), 10 $\mu$M of $MgCl_2$, 10 $\mu$M of $\beta$-mercaptoethanol, 2 $\mu$M of KF, 3 $\mu$M of dipotassium ATP (pH 6.8), 2 $\mu$M of L-amino acid, 1 $\mu$M of pyrophosphate containing $10^5$ to $10^6$ CPM $P^{32}$-pyrophosphate (New England Nuclear Corp.), approximately 100 $\mu$g protein of cell extract and water to a volume of 1.0 ml (final pH 7.3). The mixture was incubated at desired temperature for 30 minutes, and the reaction was terminated by rapid chilling and addition of 0.2 ml of 25% trichloracetic acid. Next, 0.3 ml of acid-washed Norit A suspension (Fisher Sci. Co.), containing 200 mg per ml, for adsorbing adenosine phosphate, and 1.5 ml of 0.1 M sodium acetate were added with thorough mixing.After at least 10 minutes, the Norit was re-suspended and 0.5 ml of the suspension rapidly pipetted onto a membrane filter (Millipore, HAWP, 0.45 $\mu$). The filters were

rinsed with 15 ml of 0.05 M sodium acetate buffer (pH 4.5), followed by 15 ml of water, and counted in a Nuclear-Chicago thin end window, gas flow counter. The counts were corrected for self-absorption and background radioactivity using controls. Controls contained all the constituents of reaction mixture, using heated crude cell extract or without enzyme preparation, or without amino acid. Specific activity was expressed as $\mu$M PP exchanged/mg protein/hr.

## Incorporation of $^{14}$C-uracil into Cells and Nucleic Acid Pool

The method is based on the observation that nucleic acids are insoluble but short chain nucleotides and nucleosides are soluble in acid (McDonald, 1955). Exponentially growing cells were harvested and re-suspended in TSB medium, pH 7.0 at either 20°C or 30°C to a final concentration of 1 mg dry weight of cells per ml. The fresh medium was previously incubated at the desired temperatures. The growth medium was supplemented with 40 $\mu$g uracil containing a total of 1 microcurie ($\mu$c) uracil-2-$^{14}$C (New England Nuclear Corp.). During incubation, 2 ml samples were withdrawn, and the reaction stopped by rapidly cooling the cell in an ice-bath. All the following procedures were carried out at 0 - 4°C. The cold cell suspension was

immediately centrifuged at 25,000 x g for 10 minutes and the cells washed 2 more times with 2 ml aliquots of ice-cold TSB medium. The cells were then suspended in 2 ml of 5% TCA and incubated in an ice-bath for 1 hour. The suspensions were centrifuged and the supernatant collected. Two more TCA extractions were done and the supernatants were designated as acid-soluble fraction. The residue was re-suspended in 6 ml 5% TCA and designated as acid-insoluble fraction. The radioactivity of each fraction was counted in a Liquid Scintillation Counter, Mark 1 (Nuclear Chicago).

### Incorporation of $C^{14}$- Thymine into Cells and DNA

The method was similar to that described for the incorporation of $^{14}C$-uracil with the exception that the medium was supplemented with 40 μg/ml of thymine (CalBiochem) containing a total amount of 1 μc of thymine-2-$^{14}C$ (New England Nuclear Corp.) in place of the labelled uracil.

### Incorporation of $C^{14}$- leucine into Cells and Protein

The method was similar to that described for the incorpora-

tion of $^{14}C$-uracil with the exception that the medium was supplemented with 80 μg/ml of leucine (CalBiochem) containing a total amount of 1 μc of leucine-1-$^{14}C$ (New England Nuclear Corp.) in place of the labelled pyrimidine.

## Measurement of Radioactivity in Solution

One ml of samples for counting was placed in a counting vial. To this was added 10 ml of the liquid phosphor devised by Bray (1960). This was prepared by combining:

| | |
|---|---|
| Naphthalene | 60.0g |
| 2,5-diphenyloxazole (ppo) | 4.0g |
| 1,4-bis-(2-5-phenyloxalyl-benzene) (popop) | 0.2g |
| Methanol (absolute) | 100 ml |
| Ethylene glycol | 20 ml |
| p-dioxane | to 1 liter |

Radioactivity of the preparation was determined in a Nuclear-Chicago Liquid Scintillation System (Mark 1). Quenching was determined by counting an interval standard of $^{14}C$-cholesterol and the radioactivity in the samples was expressed as cpm in the sample withdrawn.

## Qualitative Detection of Nuclease Activity

The method is the diffusion plate technique of Berry and Campbell (1965). The assay mixture consisted of:

$MgCl_2.6H_2O$,0.004M; Agar, 1.5% (w/v); in 0.01M
Tris-HCl buffer (pH 8.8).

The mixture was heated to melt the agar, cooled to 45°C, 4 mg yeast RNA/ml or calf thymus DNA/ml added and the mixture poured into petri dishes and allowed to solidify. Samples to be tested for nuclease activity were added in 0.2 ml amounts to antibiotic assay cups previously embedded in the assay mixture. Plates were incubated in a moist atmosphere for 18 hours after which the plates were developed as follows:

(a) flooded with cold 10% TCA for 15 minutes, the excess acid removed, then

(b) flooded with molybdic-aminonaphthol-sulfonic acid reagent which was prepared as follows:

$(NH_4)_6Mo_7O_{24}.4H_2O$, 2.5% (w/v) in 3N $H_2SO_4$: 1 vol;

1-amino-2-naphthol-4-sulfonic acid, 0.25% (w/v), $NaHSO_3$, 15% (w/v), $Na_2SO_4$, 0.25% (w/v), aqueous : 0.4 vol; distilled water, 8.6 vol.

The reagent was mixed just before use. The plates were incubated for 15 minutes at room temperature and examined. Nuclease activity was detected by the appearance of clear, sharply-defined zones in a blue-white opaque background.

## Quantitative Measurement of RNase Activity

The hydrolysis of RNA by RNase was measured by the formation of free acid groups not precipitated in acid (McDonald, 1955). Reaction mixtures were prepared containing, in 1.9 ml, $MgCl_2$ 20 μM, Tris buffer (150 μM, pH .8.) and RNA (4 mg). Cell extract (0.1 ml) containing about 100 μg protein, or suitable amount of RNase preparation, was added to the mixture and incubated, unless otherwise stated, at 30°C for 12 hours. The reaction was terminated by the addition of 2 ml ice-cold 10% perchloric acid and the acid-soluble fraction collected by centrifugation and filtered through a Whatman No. 1 filter paper previously soaked with the cold acid. The increase in orcinol-reacting compounds (mainly ribose) in the acid soluble fraction was

measured over controls. The controls contained all the components, except that the enzyme used had been heated in boiling water for 30 minutes, or enzyme was omitted. The specific activity was expressed as $\mu$g of acid soluble material released/mg protein/hour.

## Determination of Optimum pH of RNase Activity

Optimum pH for nucleases varies from species to species. The optimum pH of RNase activity of the crude cell extracts was measured by using acetate buffer (pH 4.5 and 5.5), phosphate buffer (pH 6.5 and 7.5) and Tris-buffer (pH 8.8 and 10.0), at a concentration of 150 $\mu$M.

## The Use of Inhibitor for Measuring RNase Activity

The measurement of RNase activity with crude cell extract might be the net activity of RNA polymerase and RNase. p-chloromercuribenzoate (PCMB) is known to inhibit RNA polymerase activity completely with a concentration of $10^{-4}$M (Hurwitz, 1963), while it has no effect on RNase even at $10^{-2}$M (Nishimura, 1960). The inhibition of RNA polymerase activity in crude cell extracts was done by adding

$10^{-4}$M PCMP/ml to the reaction mixtures, and RNase activity measured and compared with controls.

## Measurement of RNA Polymerase Activity

The method for the assay of RNA polymerase measures the incorporation of an acid-soluble radioactive nucleotide into an acid-insoluble polyribonucleotide from the reaction:

$$n(\text{ATP} + \text{GTP} + \text{CTP} + \text{UTP}) \underset{\text{Mn}^{++}}{\overset{\text{DNA}}{\rightleftarrows}} (\text{AMP-GMP-CMP-UMP})_n + 4n\text{PPi}$$

To exclude incorporation due to polynucleotide phosphorylase present in the crude enzyme, the reaction was carried out in the presence of a suitable concentration of inorganic phosphate (Ochoa _et_ _al_., 1961). The measurement of RNA polymerase activity was similar to that of Hurwitz (1963). The reaction mixture, in 0.5 ml, contained potassium phosphate buffer (1 $\mu$M; pH 7.5), Tris-buffer (25 $\mu$M; pH 7.5), 2-mercaptoethanol (4 $\mu$M), $MnCl_2$ (2 $\mu$M), $C^{14}$-ATP (1.6 $\mu$M containing 2 x $10^5$ CPM, New England Nuclear Corp.), GTP, CTP and UTP (1.6 $\mu$M each), calf thymus DNA (50 $\mu$g) and crude extracts (about 100 $\mu$g of protein) or suitable amount of enzyme preparation. The mixture was incubated for 60 minutes, unless otherwise stated, at 20°C. The reaction was stopped by the

addition of 2 ml of ice-cold 5% TCA. The entire solution was filtered through a Millipore filter (0.45 $\mu$), previously soaked with cold TCA, and the reaction tube rinsed twice with 2 ml aliquots of 5% TCA which were also filtered through the millipore. The precipitate was washed again with 10 ml 5% TCA. The filters were dried with gentle heat and placed on an aluminum planchet, and the radioactivity measured in a Nuclear-Chicago thin end window, gas flow counter. The activity of RNA polymerase was compared with controls in each series of assays. The control tubes contained either no enzyme or enzyme preparation which had been heated in boiling water for 30 minutes. The specific activity of RNA polymerase is defined as millimicromole of $^{14}$C-ATP incorporated into RNA/hour/mg protein.

## Determination of Deoxyribonuclease Activity

The hydrolysis of DNA by deoxyribonuclease (DNase) was measured by the formation of free acid groups which are not precipitable in cold acid (McDonald, 1955). Reaction mixtures contained, in 1.9 ml sodium phosphate buffer (150 $\mu$M; pH 7.5), $MgCl_2$ (20 $\mu$M) and calf thymus DNA (4 mg). 0.1 ml of cell extract containing about 100 $\mu$g protein was added to the mixture and incubated at a fixed temperature for 12 hours. The reaction was terminated by the addition of 2 ml ice-cold 10%

perchloric acid. After 1 hour at 0°C the acid-soluble fraction was collected by centrifugation. Controls were carried out at each assay temperature and contained all the components except that the cell extract had been boiled for 30 minutes, or without enzyme preparation. One unit of enzyme was defined as that enzyme activity that brought about an activity increase in OD of 0.01 at 260 m$\mu$. Specific activity was expressed as units/mg protein/hr.

## IV. ISOLATION AND CHARACTERIZATION OF RNASE AND RNA POLYMERASE

The activities of RNase and RNA polymerase in cell extracts may be affected by many factors. Attempts were made to isolate and purify these two enzymes.

### Ribonuclease

Unless otherwise stated, all operations were carried out at 0 to 4°C. All centrifugations were at 25,000 x g for 10 minutes.

The cells of ATCC 15174 (8g) grown exponentially were collected and sonically disrupted as described before. The broken cell suspension was then centrifuged for 20 minutes and the supernatant fluid was collected (Fraction I). To 130 ml of cell extract, was

added, with stirring, 80 ml of a 1% protamine sulphate solution (Sigma). After standing for 10 minutes, the suspension was homogenized with 160 ml of Tris buffer, 0.1M (pH 8.8) for 5 minutes at one-fourth of maximal power of a 10-KC Branson Sonifier, model S-75. The suspension was centrifuged, and the resulting precipitate homogenized with 160 ml of Tris buffer, 0.5M, pH 8.8 for 10 minutes, then centrifuged as before. To the supernatant fluid was added an equal volume of distilled water and 1 ml of 0.5M $MgCl_2$. The diluted solution was incubated at 37°C for 2 hours. A heavy precipitate of inactive protein which formed during incubation was removed by centrifugation and the supernatant collected (Fraction II). With stirring, 102g of solid ammonium sulphate was added to 320 ml of protamine eluate. The suspension was allowed to stand for 30 minutes after the ammonium sulphate had dissolved, and then centrifuged. To the supernatant solution was added, with stirring, an additional 113g of ammonium sulphate. The suspension was allowed to stand and centrifuged again. The resulting precipitate was dissolved in 16 ml of Tris buffer, 0.02M (pH 8.8). The solution was dialyzed for 12 hours against 2 liters of Tris, 0.01M (pH 8.8). An inactive precipitate which formed upon dialysis, was removed by centrifugation and the supernatant collected (Fraction III). A column of DEAE-cellulose (16 x 2.2cm) was prepared according to the method of Peterson and Sober (1962) and equilibrated with 2 liters of Tris,

0.01M, pH 8.8. The dialyzed ammonium sulphate fraction was applied to the column at the rate of 60 ml/hr and eluted with a linear gradient of 0.0 to 0.75M NaCl in 0.01M (pH 8.8). Ten ml fractions were collected and assayed for protein content and RNase activity. The active fractions came out between 0.2 to 0.3M NaCl. Active fractions were pooled, dialyzed against Tris buffer, 0.01M (pH 8.8), and the precipitate removed by centrifugation. The supernatant was concentrated in a Virtis Freeze Dryer (Virtis Res. Eqn., N.Y.) over night. The concentrate was dissolved in 6 ml Tris, 0.02M, pH 8.8 and the inactive protein removed (Fraction IV).

The effect of pH and temperature on the activity of the purified RNase was determined as before.

## RNA Polymerase

The properties of RNA polymerase activity in ATCC 15174 are quite obscure. Attempts to purify the enzyme were therefore made according to the procedures of Hurwitz (1963).

Operation temperature, centrifugation and procedures up to the collection of cell extracts were same as for RNase purification. To 170 mg protein of cell extract in 200 ml solution containing

0.01M Tris (pH 7.5), 0.01M $MgCl_2$ and 0.001M mercaptoethanol was treated with 50 ml of a 0.5% protamine sulphate solution. After 5 minutes at 0°C, the stringy precipitation was collected by centrifugation at 6,000 x g. The pellet was homogenized as before with 100 ml of a solution containing 0.1M Tris (pH 7.5) and 0.001M mercaptoethanol. RNA polymerase was then eluted from the protamine precipitate by homogenizing with 130 ml of a solution containing 0.5M succinate buffer (pH 7.5), and 0.001M mercaptoethanol to yield protamine eluate. The protamine eluate was adjusted to 20% saturation with solid ammonium sulphate and the precipitate removed by centrifugation. The supernatant solution was then adjusted to 45% saturation of solid ammonium sulphate. The precipitate was dissolved with 10 ml of 0.01M Tris (pH 7.5) containing 0.001M mercaptoethanol and dialyzed against 2 liters of the buffer for 12 hours. The precipitated protein was removed by centrifugation. The column of DEAE-cellulose was equilibrated with 2 liters of 0.01M Tris (pH 7.5) containing 0.001M mercaptoethanol. The dialyzed ammonium sulphate fraction was applied to the column at the rate of 60 ml/hour and eluted with a linear gradient of 0.0 to 0.75M NaCl in 0.01M Tris (pH 7.5) containing 0.001M mercaptoethanol. Ten ml fractions were collected and assayed for protein content and enzyme activity. The

"active fractions" were pooled, dialyzed and concentrated as in RNase purification.

## Determination of Enzyme Activity

Determination of RNase activity was based on that of McDonald (1955) and detection of RNase activity from column fractions was that of Berry and Campbell (1965), all assayed at 30°C. RNA polymerase activity was measured by the method of Hurwitz (1963) at 20°C as described previously.

## Estimation of Protein Content

The protein content was measured either by the phenol method of Lowry *et al*. (1951) or the optical method of Warburg and Christian (1941), the latter applying only to samples after cellulose purification.

# RESULTS

## I. GENERAL

A knowledge of the basic characteristics of an organism is an important prerequisite for further study. These characteristics were determined for Microcuccus cryophilus at the start of this research programme and proved to be helpful in the experimental design of subsequent research.

Micrococcus cryophilus ATCC 15174 is an unusually large coccus occurring in chains, clusters and diploforms and as single spheroid cells. These cells, under optimum conditions, were found to vary in size, averaging 1.6 μ diameter with large cells measuring 3.6 μ in diameter. They are non-motile and aerobic. Gram stains showed Gram-positive cells with a predominant number of Gram-negative cells irrespective of the age of the culture. Tests for gelatin liquefaction, production of rennet, indole and hydrogen sulphide, starch hydrolysis, and ability to reduce nitrates were all negative, as was haemolysis. The organism is lysed by sodium lauryl sulphate and partially lysed by lysozyme. The cells tolerate

high salt concentration (7.5% NaCl) but cannot grow in the presence of penicillin. It is not capable of growth in sodium citrate or in $NH_4H_2PO_4$ as a sole source of carbon and nitrogen respectively. With 24 carbohydrates tested, including glucose, fructose, lactose, sucrose and maltose, none showed the production of acid. MR-VP test was negative. Urease, catalase and oxidase were formed but no extracellular nuclease could be detected. Glutamate can be used as sole carbon and nitrogen source. Growth occurs from 0°C to 24 to 25°C and is optimal at 20°C. Optimum pH for growth is 7.2, but growth occurred in the range of 5.5 to 9.5. The organism showed a faint pink in colour on agar plates, the colour being enhanced as the incubation temperature decreased.

## Selection of Mutants

After cultures of ATCC 15174 had been irradiated with ultraviolet light, several mutants developed with the ability to form colonies at 30°C. The mutation rate was about $10^{-7}$ to $10^{-8}$. Of the mutants with elevated growth temperatures, T8 and M19 were found to have basically the same physiological characters as the parent. The main differences between mutants and their parent are

shown in Table 1. It is of interest that the mutants possess a wide temperature range of growth with a maximum at 34°C (T8) and 37°C (M19). However, since the mutants cannot form colonies on agar medium at 0°C within 2 weeks, they may be better designated as mesophiles rather than psychrophiles (Ingraham and Stokes, 1959). The mutants gain the ability to reduce nitrate, but show a decrease or loss of oxidase activity, and grow poorly, only forming tiny colonies on glutamate minimal medium. These changes in activity suggest changes in the metabolic pathways resulting in a requirement for some nutrients in addition to, or replacing, glutamate to support vigorous growth at elevated temperatures. No special attempts were made to study these changes in the nutritional requirements of the mutants. To obtain the cells of the parent organism and mutants with comparable growth rates, the complex medium was used thereafter.

## Transformation of Growth Temperature Response

Cultures of ATCC 15174 with the addition of purified DNA of T8 and M19 produced many phenotypic colonies at 30°C (Table 2). The transformation frequency was about $10^{-4}$. Twenty minutes of

TABLE 1. Main Differences of General Properties of Mutants, T8 and M19, and Their Parent, ATCC 15174.

| Strain | ATCC 15174 | T8 | M19 |
|---|---|---|---|
| Growth range | -4 - 24°C* | 4 - 34** | 4 - 37** |
| Optimum growth temperature | 20°C | 30°C | 30°C |
| Utilization of glutamate | ++ | + | + |
| Catalase | + | ++ | ++ |
| Oxidase | ++ | - | + |
| Nitrate reduction | - | + | + |
| Colony colour | light pink | creamy | pink |

* McLean _et_ _al_. (1951).

** No colony formed at 0°C within 2 weeks.

TABLE 2. Number of $30^{+}$ Transformants obtained from Treated Recipient Population (ATCC 15174)

| Treatment | No. of Transformants |
|---|---|
| 1. ATCC 15174 control* | 0 |
| 2. T8 DNA preparation | 1,000 |
| 3. M19 DNA preparation | 1,100 |
| 4. ATCC 15174 DNA preparation | 0 |
| 5. Calf Thymus DNA | 0 |
| 6. T8 DNA Digest** | 30 |
| 7. M19 DNA Digest** | 42 |

* Control cells incubated at 20°C showed $2 \times 10^{7}$ cells/ml

** DNase (50 μg/ml) and DNA preparation (40 μg/ml) were added together and incubated for 60 minutes at 20°C.

reaction between donor DNA and the recipient seemed to be enough to reach the maximal transformation number. The number of transformants was greatly reduced when the DNA was treated with DNase (Table 2, assays 6 and 7), as the biologically active DNA was destroyed. When these colonies were replicated, they grew well on glutamate minimal medium at 20°C, however, about 90% lost their ability to grow at 30°C even on the complex medium. It is possible that DNA polymers protected the cells of ATCC 15174 temporarily to form the phenotypic colonies at 30°C. However, this was eliminated by the controls containing the same amount of DNA extracted from ATCC 15174 or calf thymus under the same conditions, both of which did not bring about the formation of colonies. The mechanism of the transformation and the reasons why 90% of the transformants lost the ability to grow at 30°C after multiplying for several generations to form a colony are unknown.

## Denaturation Temperature and Base Composition of DNA

The Tm of DNA of ATCC 15174 and M19 in 0.15 NaCl - 0.015M sodium citrate solution (pH 7.2) were found to be 84.5°C and 85°C respectively (Fig. 5). From these data, DNA lability would appear not to be related to maximum growth temperature as indicated by

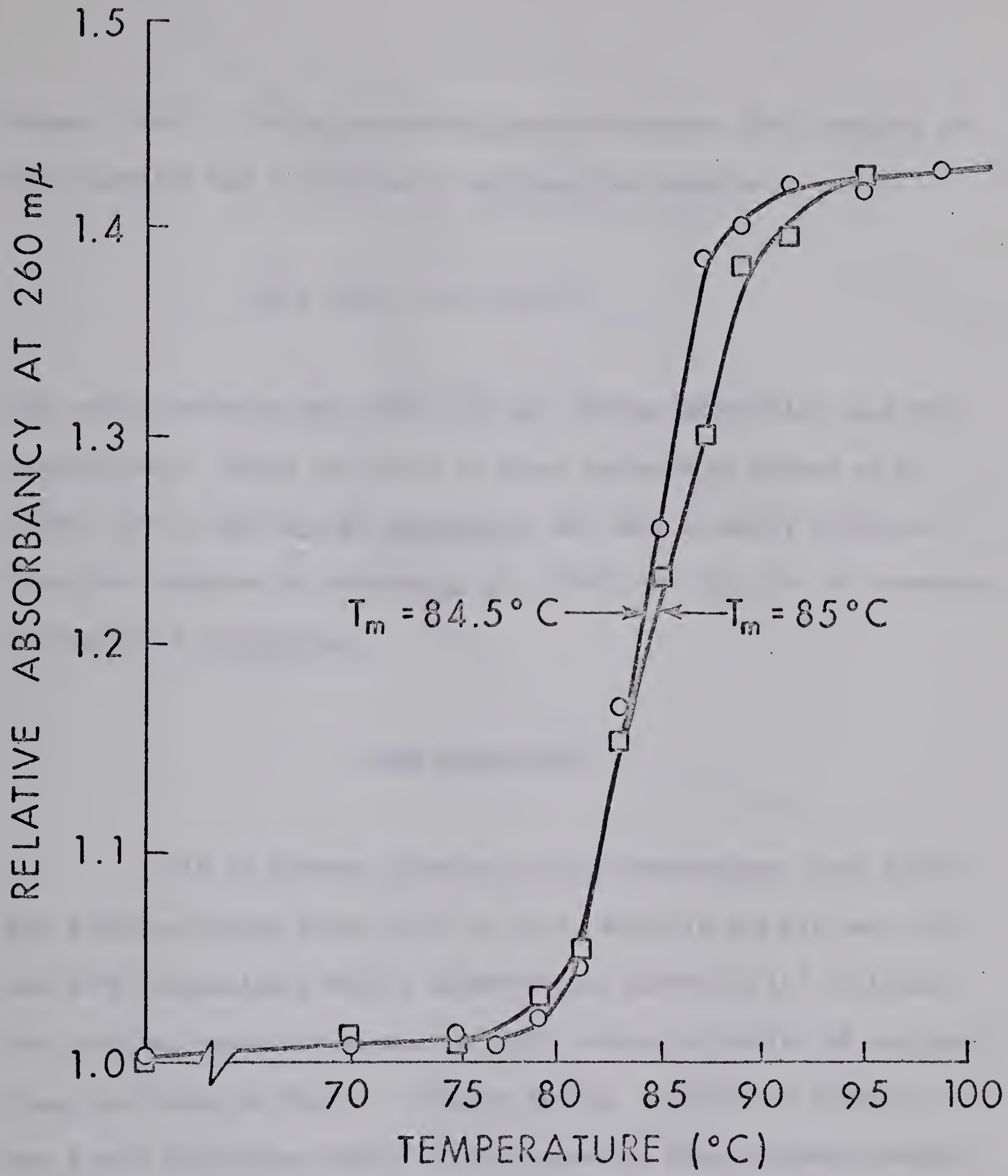

Fig. 5 Denaturation temperature (Tm) of DNA of ATCC 15174 and M19

○——○ ATCC 15174
□——□ M19

Marmur (1961). The corresponding Guanine-Cytosine (G-C) content of the organisms was calculated by applying the equation:

$$Tm = 69.3 + 0.41\ (G\text{-}C)$$

The values obtained were about 37% and 38% for ATCC 15174 and M19 respectively. These are close to those reported by Marmur *et al*. (1963) (38 to 40%) for *M. cryophilus* and only slightly different from that reported by Bohacek *et al*. (1967) (41.3%), for an organism designated *M. cryophilus*.

## Heat Resistance

With 10 minutes exposure to high temperature, ATCC 15174 had a Thermal Death Point (TDP) at 55°C, while T8 and M19 were 75°C and 65°C respectively with a concentration factor of $10^7$ cells/ml. The survival curves of mutants at 55°C, which is the TDP of the wild type, are shown in Fig. 6. Complex medium, as would be expected, has a more protective effect on the organisms than phosphate buffer. T8 with a maximum growth temperature (MGT) at 34°C is more heat-resistant than M19 (MGT at 37°C) under the same conditions, in terms of both TDP or D values, the latter being the time required to reduce

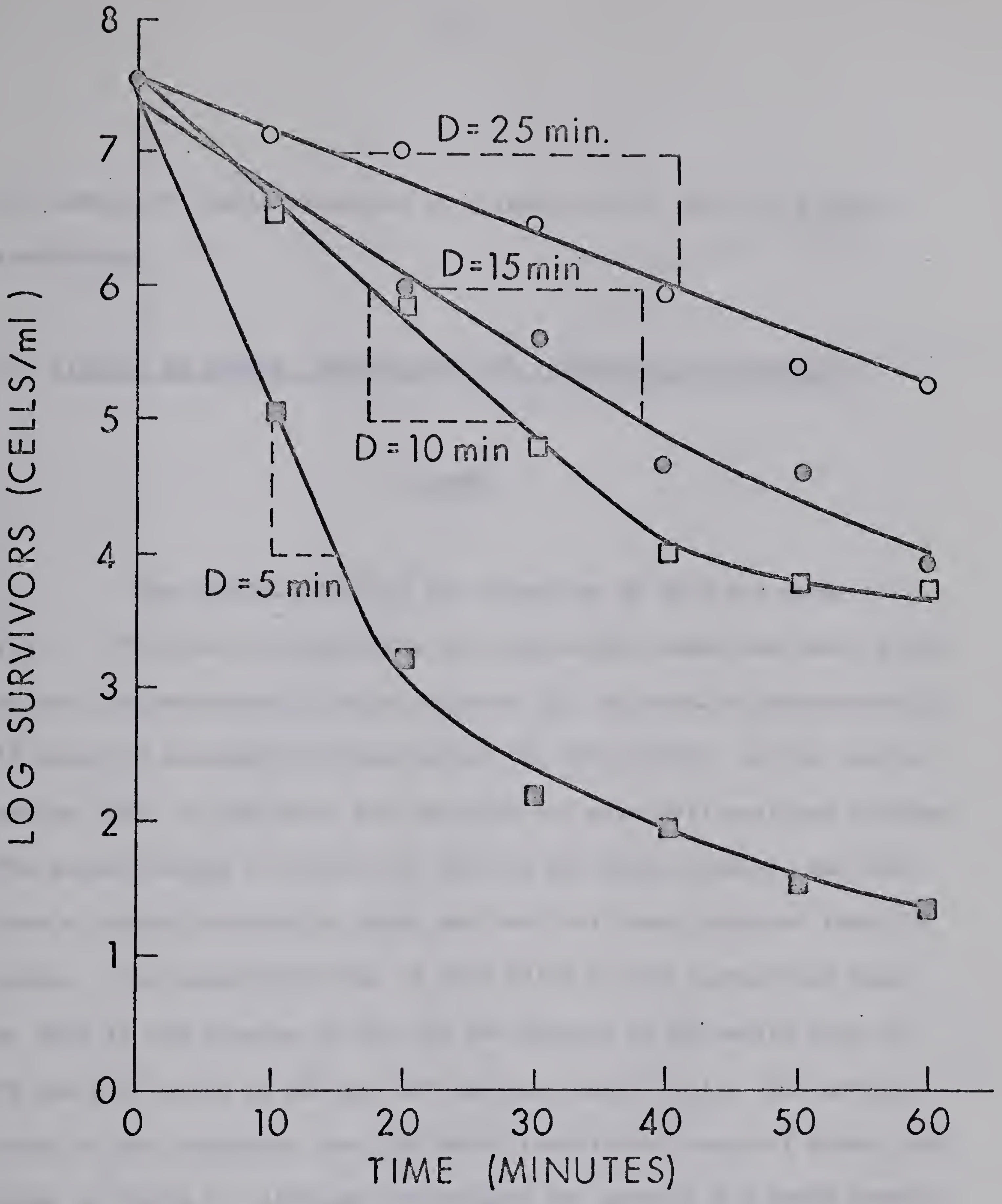

Fig. 6 Survival of mutants at 55° C

○——○ T8, TSB medium
●——● T8, phosphate buffer
□——□ M19, TSB medium
■——■ M19, phosphate buffer

the number of viable organisms by a factor of 10 (90%) at a given temperature.

## II. STUDIES ON GROWTH, RESPIRATION AND INTERMEDIARY METABOLISM

### Growth

The growth curves of the organisms at 20°C are shown in Fig. 7. Four sub-cultures from the exponential phase had taken place before the measurements began reducing the lag phase to approximately 15 hours in glutamate minimum medium for ATCC 15174. In the complex medium, TSB, no lag phase was observed and more cell mass was produced. The growth curves of mutants T8 and M19 are quite similar, and both have a longer exponential phase and more cell mass produced than the parent. The generation time of ATCC 15174 in the exponential phase at 20°C is 150 minutes in TSB and 240 minutes in MM, while that of T8 and M19 in TSB is 220 and 240 minutes respectively. The growth rates of the organisms over the whole temperature range of growth are shown in Table 3. Although the mutants can grow at 0°C their generation time is about 55 hours and therefore they should not be considered as psychrophiles (Ingraham and Stokes, 1959).

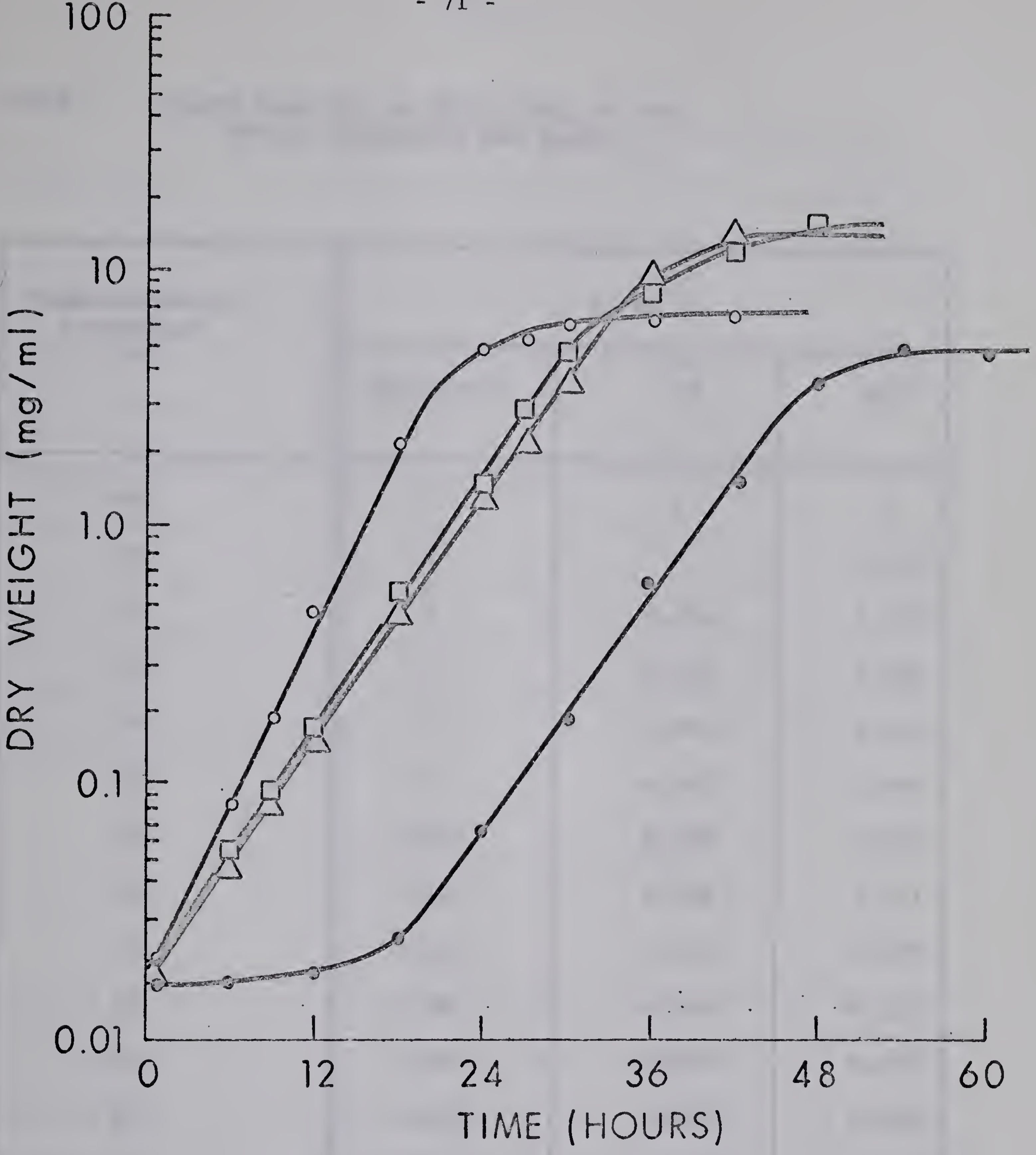

Fig. 7 Growth of ATCC 15174, T8 and M19 at 20° C

○——○ ATCC 15174, TSB
●——● ATCC 15174, mm
□——□ T8 TSB
△——△ M19 TSB

TABLE 3 Growth Rate (k) of ATCC 15174, T8 and M19 in Trypticase Soy Broth

| Temperature of Incubation °C | k (hr$^{-1}$) | | |
|---|---|---|---|
| | ATCC 15174 | T8 | M19 |
| 40 | * | * | * |
| 37 | * | * | 0.143 |
| 34 | * | 0.154 | 0.297 |
| 32 | * | 0.231 | 0.396 |
| 30 | * | 0.362 | 0.416 |
| 27 | * | 0.333 | 0.346 |
| 24 | 0.231 | 0.320 | 0.297 |
| 20 | 0.287 | 0.188 | 0.173 |
| 17 | 0.238 | 0.173 | 0.139 |
| 14 | 0.185 | 0.116 | 0.112 |
| 12 | 0.160 | 0.099 | 0.097 |
| 10 | 0.148 | 0.069 | 0.066 |
| 7 | 0.092 | 0.042 | 0.040 |
| 4 | 0.050 | 0.021 | 0.021 |
| 0 | 0.024 | 0.012 | 0.012 |

* No growth observed

[illegible]

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The effect of temperatures on growth of the organisms is shown in Fig. 8. The principal differences between the psychrophile and the mesophiles is that the maximum growth temperature and the minimum growth temperature of the former are 10 - 13°C and 4 - 5°C, respectively, below those of the latter. The shape of the curves for the two classes of bacteria appears to be quite similar. However, when an Arrhenius type plot is applied, a more striking difference becomes apparent (Fig. 9). The slopes of the linear portion of the descending curves differ for the psychrophile and mesophilic mutants by a factor of about 1.5. The slope of this part of the curve is the temperature characteristic ($\mu$) and its numerical value in calories is calculated from the formula:

$$\text{rate} = Ae^{-\mu/RT} \quad \text{or} \quad \ln \text{rate} = -\mu RT + C$$

where A is the entropy constant, R is the gas law constant and T is the absolute temperature. The value of $\mu$ for ATCC 15174 is 10,100 whereas those of T8 and M19 are 16,100 and 15,000 respectively. It can be seen that $\mu$ is markedly less for the psychrophile than for the mesophiles. Thus, the amount of activation energy required in a given temperature range is much less for the psychrophile than for

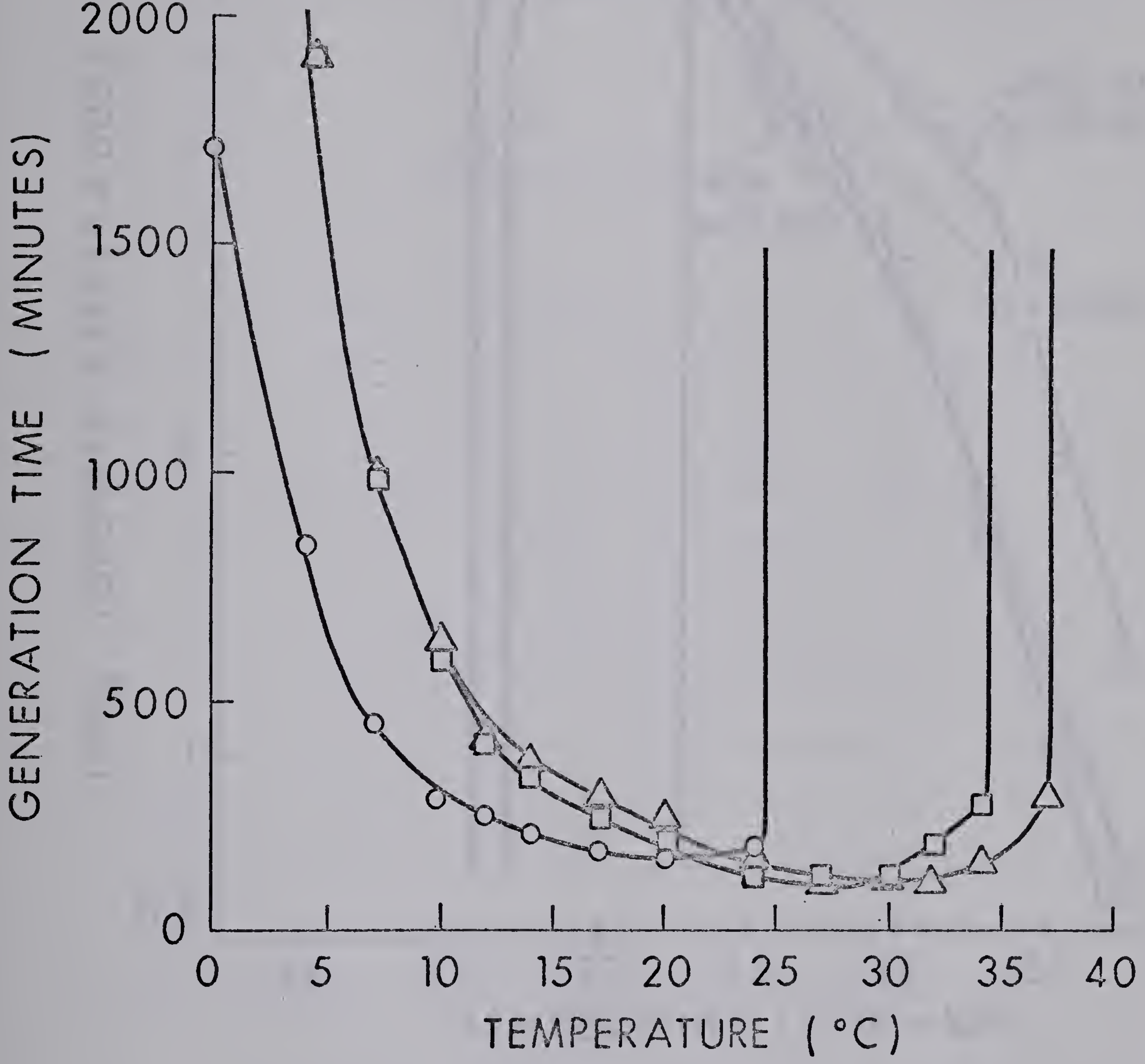

Fig. 8 Effect of Temperature on the generation time of ATCC 15174, T8 and M19

○————○ ATCC 15174
□————□ T8
△————△ M19

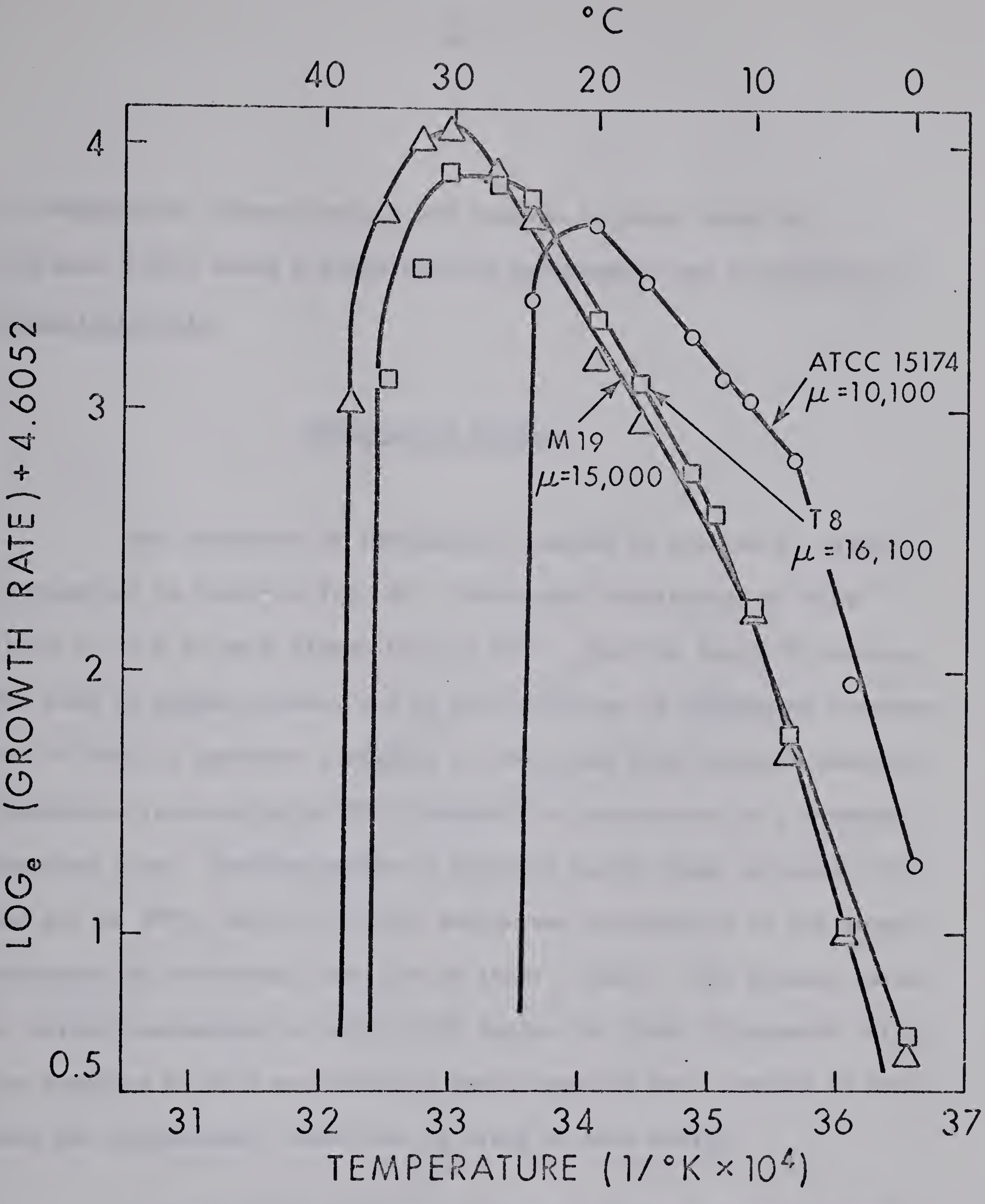

Fig. 9 Arrhenius plot of the relationship between growth rate and temperature

| | |
|---|---|
| ○———○ | ATCC 15174 |
| □———□ | T8 |
| △———△ | M19 |

the mesophiles. These results are similar to those found by Ingraham (1958) using a psychrophilic pseudomonad and a mesophilic *Escherichia coli*.

## Respiratory Studies

The breakdown of endogenous reserves as studied by oxygen consumption is shown in Fig. 10. Endogenous respiration of ATCC 15174 at 30°C is much higher than at 20°C. For the first 20 minutes, the rate of oxygen uptake, due to the oxidation of endogenous reserves, can be seen to approach a maximum at 30°C, and then decrease greatly. Endogenous respiration at 20°C, however, is maintained at a somewhat constant rate. The phenomenon is found to be the same in mutants T8 and M19 at 20°C, but at 30°C the endogenous respiration of the mutants continues at a constant rate for at least 2 hours. The greater rates of oxygen consumption of ATCC 15174 during the first 20 minutes after the exposure to 30°C may indicate the attempt of the organism to overcome the unfavourable condition by using up more energy.

Although ATCC 15174 cannot grow at 30°C, no drastic change of oxygen consumption occurred at 30°C in the presence of glutamate (Fig. 11). However, oxygen uptake at 20°C was greater than at 30°C.

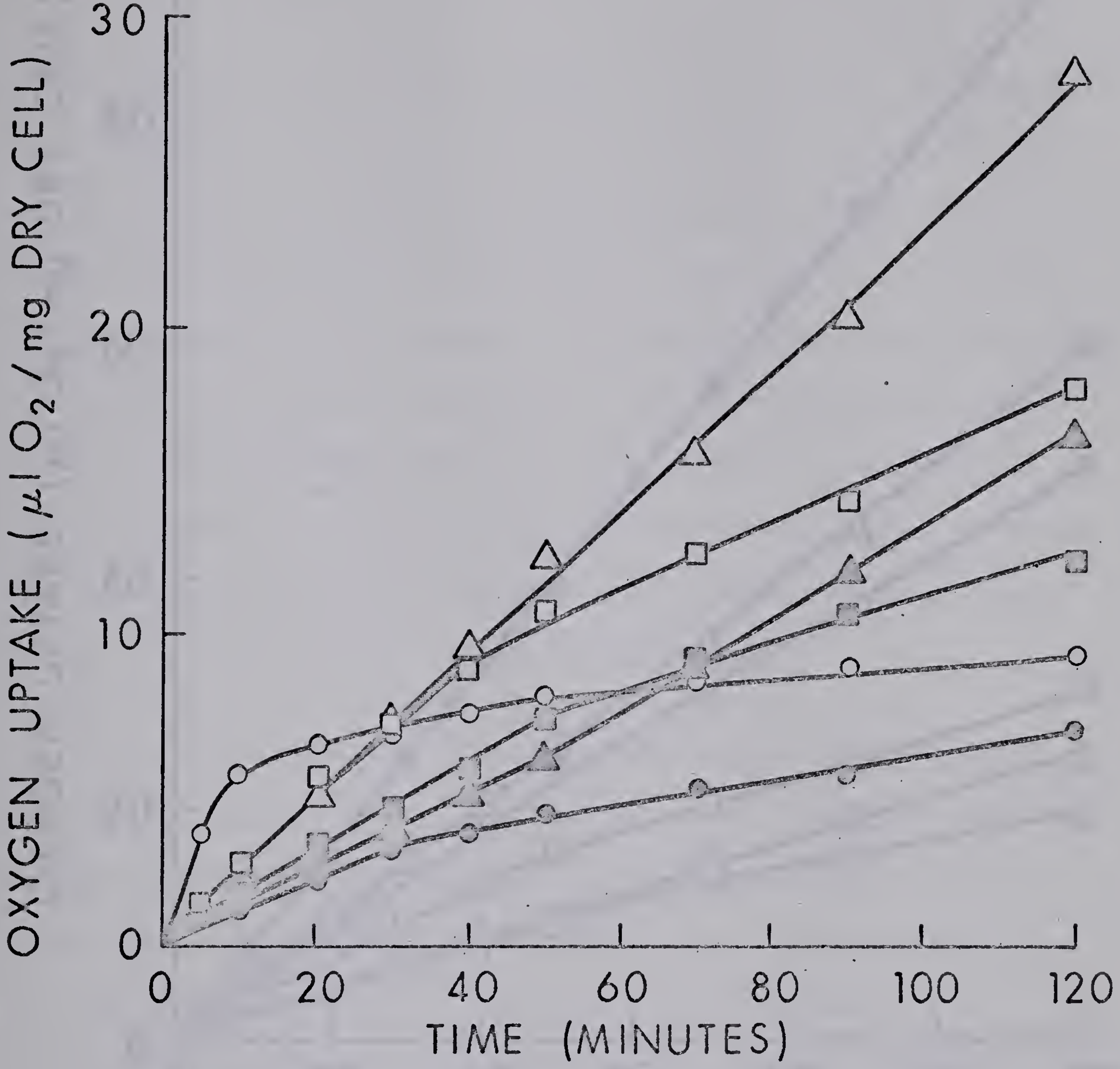

Fig. 10 Endogenous Respiration of ATCC 15174, T8 and M19 at 20° C and 30° C

○——○ ATCC 15174, 30° C
□——□ T8 30° C
△——△ M19 30° C

●——● ATCC 15174, 20° C
■——■ T8 20° C
▲——▲ M19 20° C

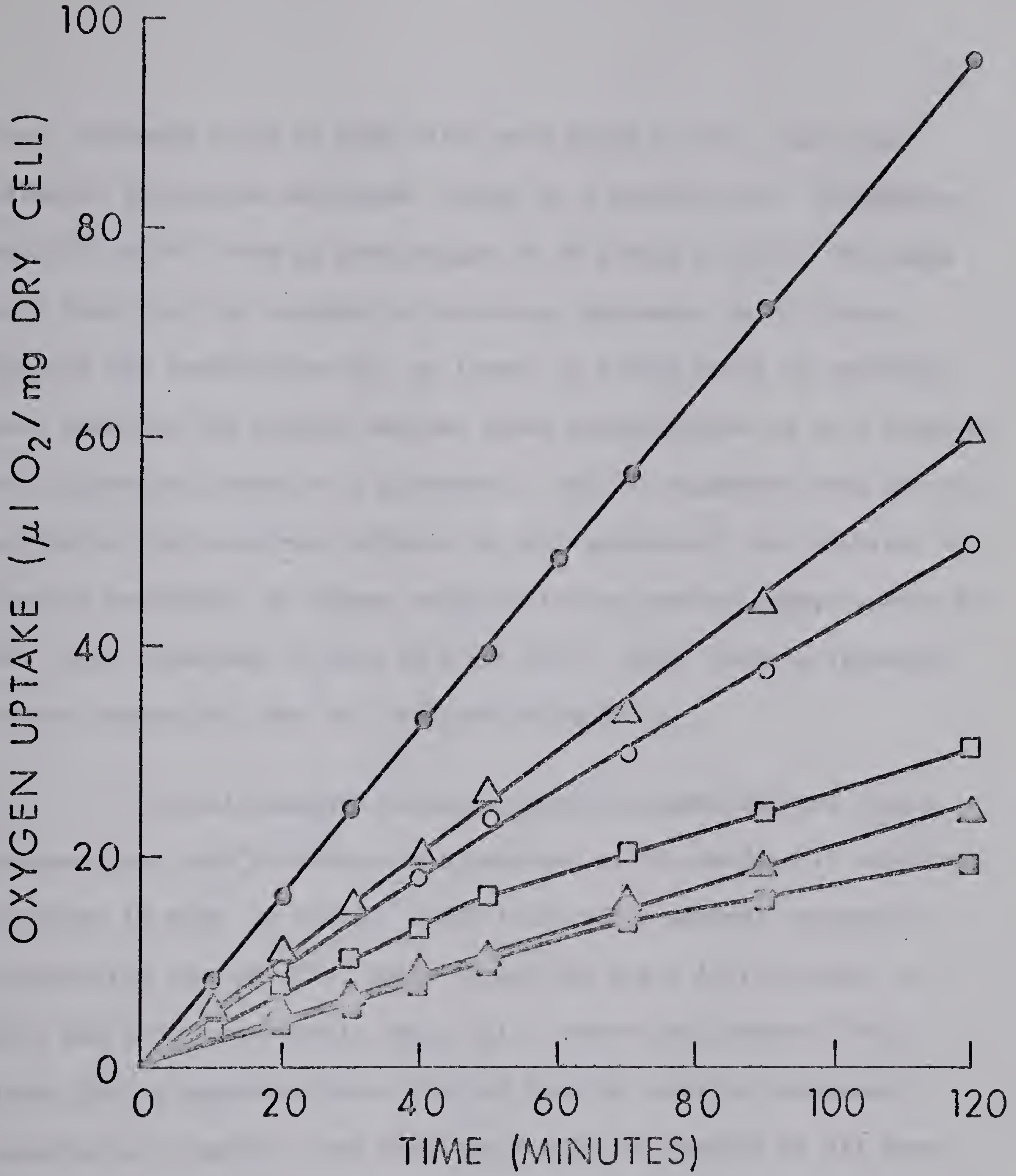

Fig. 11 Oxygen Uptake in the presence of glutamate at 20° C and 30° C (without the correction of endogenous respiration)

| | | | | | |
|---|---|---|---|---|---|
| ○———○ | ATCC15174, | 30° C | ●———● | ATCC 15174, | 20° C |
| □———□ | T8 | 30° C | ■———■ | T8 | 20° C |
| △———△ | M19 | 30° C | ▲———▲ | M19 | 20° C |

Thus, although cells of ATCC 15174 were dying at 30°C, their respiratory activities continued, though at a lesser rate. In contrast, both T8 and M19 took up more oxygen at 30°C than at 20°C. The data also show that the enzymes for oxidizing glutamate in all three strains are constitutive or, at least, in a high level of activity when grown in the complex medium, since oxygen uptake is at a constant rate after the addition of glutamate. This is supported from the observation that upon the addition of chloramphenicol, an inhibitor of protein synthesis, no change occurred in the rate of oxygen uptake by all three organisms at both 20°C and 30°C. Thus, protein synthesis is not responsible for the observed differences.

The relationship between reaction temperature and oxygen consumption, with or without the presence of the oxidizable substrate, is shown in Figs. 12 and 13. ATCC 15174 has a maximal endogenous respiration rate at 25°C, while T8 and M19 are a little higher at 28°C and 37°C respectively (Fig. 12). Baxter and Gibbons (1962) found that a psychrophilic strain of Candida respired endogenous reserves at a greater rate than the mesophiles studied at all temperatures tested. This is not the case with the organisms studied here, mutants T8 and M19 respiring even more endogenous reserves than their psychrophilic parent. Above the optimum growth temperature, the $QO_2$ of ATCC 15174 decreased as the temperature increased in the

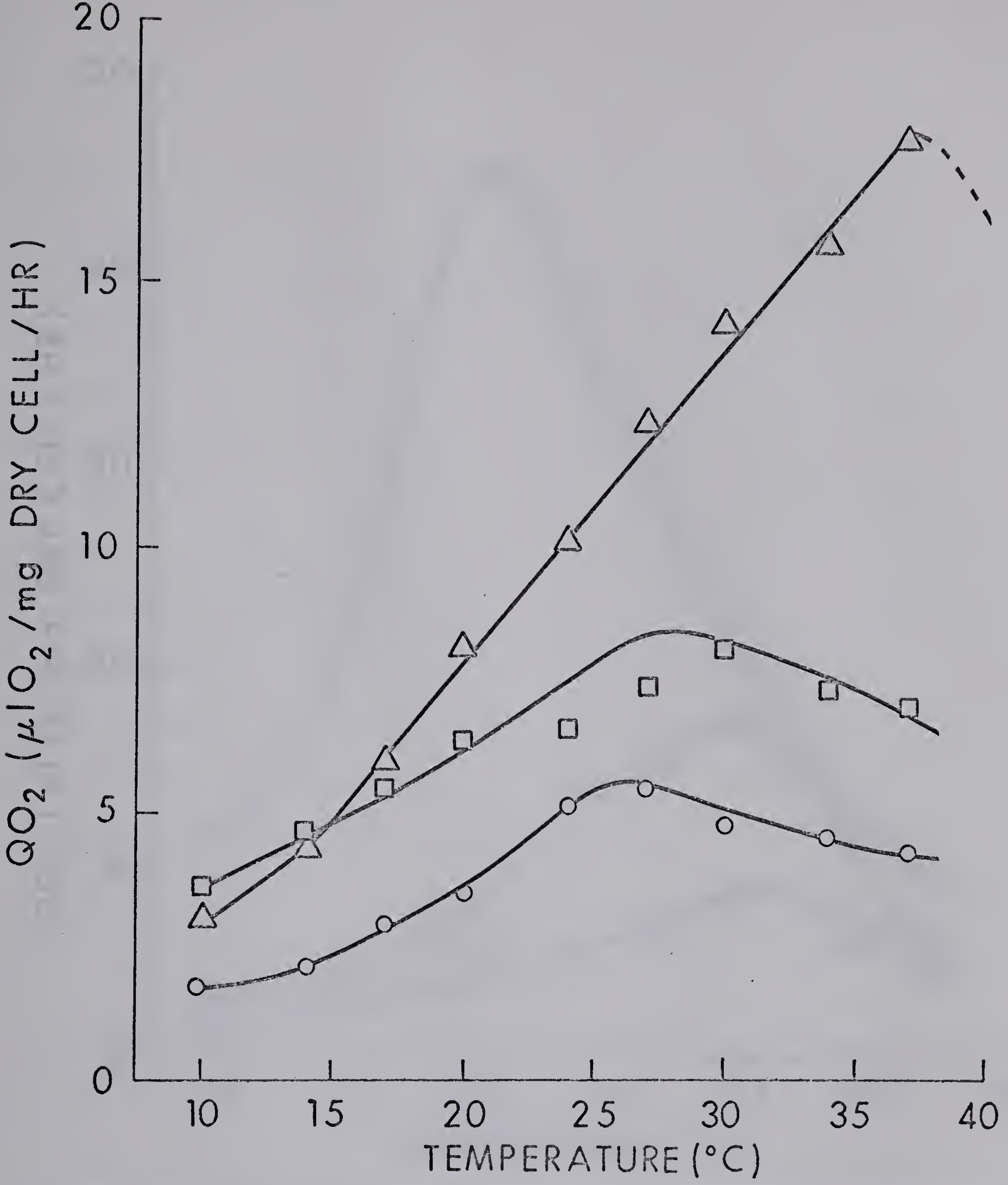

Fig. 12 Effect of temperature on endogenous respiration

○————○ ATCC 15174
□————□ T8
△————△ M19

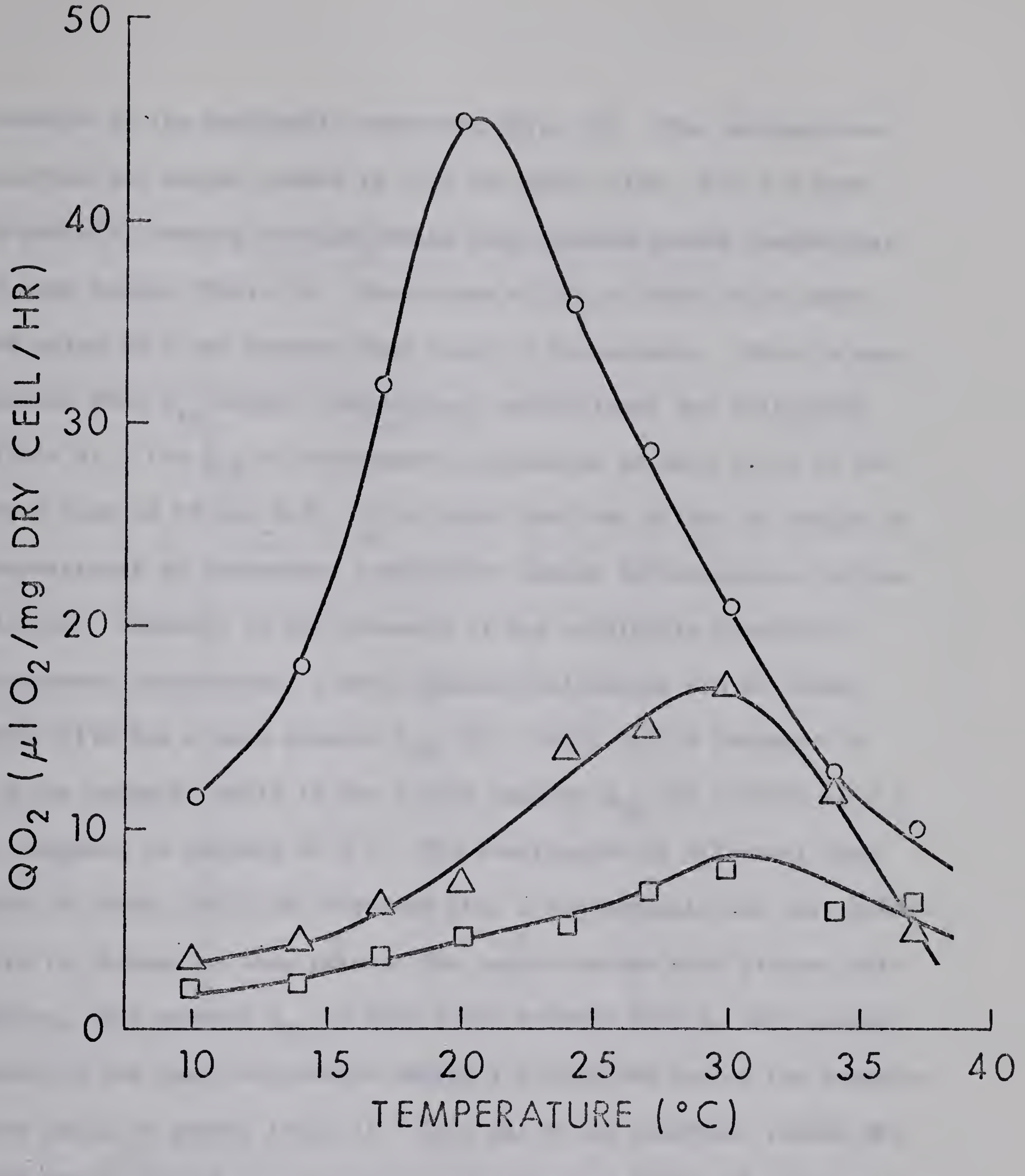

Fig. 13 Respiration rate at different temperature in the presence of glutamate

○———○ ATCC 15174
□———□ T8
△———△ M19

presence of the oxidizable substrate (Fig. 13). The optimum temperature for oxygen uptake is 20°C for ATCC 15174, 30°C for both T8 and M19, showing a relationship with optimum growth temperature of each strain (Table 3). The slopes of $QO_2$ of ATCC 15174 above and below 20°C are greater than those of the mutants. This is more obvious when $Q_{10}$ values (temperature coefficient) are calculated (Table 4). The $Q_{10}$ of endogenous respiration of ATCC 15174 is between that of T8 and M19. It is clear that the effect of change of temperatures on endogenous respiration cannot differentiate the two classes. However, in the presence of the oxidizable substrate (exogenous respiration) a more apparent difference can be found. ATCC 15174 has a much greater $Q_{10}$ (10 - 20°C) of 3.8 compared to 2.2 for mutants, while it has a much smaller $Q_{10}$ (20 - 30°C) of 0.5 as compared to mutants of 2.1. The observation is different from that of Brown (1957) who reported that a psychrophile was less sensitive to changes in temperatures for oxygen uptake with glucose oxidation. The greater $Q_{10}$ of ATCC 15174 between 10°C to 20°C corresponds to the lower activation energy ($\mu$) required during the temperature range of growth (Fig. 9). This may be an important reason why ATCC 15174 grows better than the mutants below 20°C. If the total response of $Q_{10}$ is accepted with the reference temperatures set at

TABLE 4 Temperature Coefficients of ATCC 15174, T8 and M19 with and without Glutamate Oxidation

| | Endogenous | | | Exogenous* | | |
|---|---|---|---|---|---|---|
| | ATCC 15174 | T8 | M19 | ATCC 15174 | T8 | M19 |
| $Q_{10}$ (10°C - 20°C) | 2.2 | 1.7 | 2.6 | 3.8 | 2.2 | 2.3 |
| $Q_{10}$ (20°C - 30°C) | 1.5 | 1.3 | 1.7 | 0.5 | 2.0 | 2.1 |
| $Q_{10}$** (10°C - 30°C) | 1.7 | 1.5 | 2.1 | 1.3 | 2.1 | 2.2 |

* with the correction of endogenous respiration

$$** \ Q_{10} = \sqrt{\frac{QO_2 \text{ at } 30°C}{QO_2 \text{ at } 10°C}}$$

10°C and 30°C (Ingraham and Bailey, 1959), both mesophilic mutants have a $Q_{10}$ about 2.1 while the psychrophilic parent is about 1.3, only about two-thirds that of mesophilic mutants. The growth processes of an obligate psychrophile may thus be characterized by a lower temperature coefficient ($Q_{10(10\ -\ 30^\circ C)}$) than that of mesophiles.

## Studies of Enzymes of Intermediary Metabolism

All enzymes of Kreb's cycle were detected as recorded in Table 5. Malcolm (1967) reported no deleterious effect of high temperatures on these enzymes of ATCC 15174. The comparison of enzyme activities of the organisms was therefore done at 20°C, at which temperature all three strains grow well. There is no glyoxylate cycle present in any strain. The specific activity of most enzymes among the strains is different (Table 5). This may indicate the change of catabolic pathways of the mutants while gaining the character of growing at higher temperatures. Since the utilization of glutamate by ATCC 15174 has been demonstrated to be via transaminase and then to Kreb's cycle (Malcolm, 1967), the decrease of activity of glutamate-oxaloacetate transaminase in mutants may be an important factor responsible for their poor growth and smaller

TABLE 5. Activity of Intermediary Enzymes in ATCC 15174, T8 and M19 at 20°C.

| | Specific Activity ($\mu$M substrate formed/mg protein/hr | | |
|---|---|---|---|
| | ATCC 15174 | T8 | M19 |
| Citrate synthetase | 4.1 | 6.7 | 6.1 |
| Aconitate hydratase | 3.8 | 2.0 | 5.6 |
| Isocitrate dehydrogenase | 7.2 | 16.8 | 15.3 |
| Alpha-ketoglutaric dehydrogenase | 3.8 | 3.0 | 2.1 |
| Succinic dehydrogenase | 11.0 | 2.5 | 5.5 |
| Fumerase | 2.1 | 0.2 | 2.2 |
| Malic dehydrogenase | 21.8 | 16.5 | 36.3 |
| Pyruvic dehydrogenase | 1.2 | 2.3 | 2.7 |
| Isocitratase | 0 | 0 | 0 |
| Malic synthetase | 0 | 0 | 0 |
| Glutamic dehydrogenase (NADH . $H^+$ - linked or NADPH . $H^+$ - linked) | 0 | 0 | 0 |
| Glutamic-oxaloacetic transaminase | 2.6 | 0.6 | 0.8 |

amount of oxygen uptake on glutamate.

## III. STUDIES OF MARCROMOLECULAR SYNTHESIS AND BREAKDOWN

Malcolm (1967) reported that both amino acids and adenosine-5'-triphosphate were found to accumulate in ATCC 15174 at 30°C, suggesting an inhibition of macromolecular synthesis. The transfer-RNA synthetases of alanine, aspartic acid, glutamic acid, histidine, phenylalanine and tryptophan were observed to have reduced activity at 30°C as compared with 20°C, as measured by the hydroxamate method of Stulberg and Novelli, (1962).

When the activities of these six amino-acyl-t-RNA synthetases, together with that of leucine, were assayed, no such differences were found (Table 6). However, the hydroxamate method of measuring the enzyme activities is not very sensitive. Therefore a pyrophosphate exchange method was applied. The possible Pi-ATP exchange was eliminated by using phosphate buffer (Stulberg and Novelli, 1962). As shown in Table 7, most of the enzyme activities are even higher at 30°C than at 20°C. The assays of cell extract which had been subjected to 30°C for 6 hours and lost 80% viability before the disruption of cells, supported the finding that there is no inactivation of amino-acyl-t-RNA synthetases at 30°C in ATCC 15174. The

TABLE 6 Amino Acyl-tRNA Synthetase Activity of Cell Extracts of ATCC 15174. (Hydroxamate Method).[†]

| Amino acid | Enzyme activity ($\mu$M hydroxamate formed/mg protein/hr) | | |
|---|---|---|---|
| | 20°C assay | 30°C assay | 30°C* heated |
| Alanine | 0.18 | 0.20 | 0.20 |
| Aspartic acid | 0.08 | 0.10 | 0.12 |
| Glutamic acid | 0.26 | 0.26 | 0.24 |
| Histidine | 0.16 | 0.20 | 0.16 |
| Phenylalanine | 0.10 | 0.14 | 0.10 |
| Tryptophan | 0.14 | 0.18 | 0.16 |
| Leucine | 0.08 | 0.12 | 0.10 |

* Cells of ATCC 15174 growing exponentially at 20°C were subjected to 30°C for 6 hours (20% survival) before the sonic disruption of cells. The assay was carried out at 30°C.

[†] All data are the average of 3 samples.

TABLE 7 Amino Acyl-tRNA Synthetase Activity of Cell Extracts of ATCC 15174 and M19 (Exchange Method).†

| Amine Acid | ATCC 15174 | | | M19 | |
|---|---|---|---|---|---|
| | Enzyme activity (μM pyrophosphate exchanged/mg protein/hr.) | | | Enzyme activity (μM pyrophosphate exchanged /mg protein/hr.) | |
| | 20°C assay | 30°C assay | 30°C heated | 20°C assay | 30°C assay |
| Alanine | 1.74 | 1.95 | 1.75 | 1.59 | 1.71 |
| Aspartic Acid | 0.99 | 1.3. | 1.03 | 1.21 | 1.16 |
| Glutamic Acid | 1.75 | 1.92 | 1.67 | 1.20 | 1.77 |
| Histidine | 1.51 | 2.11 | 1.80 | 1.48 | 1.70 |
| Phenylalanine | 1.64 | 1.79 | 1.69 | 1.56 | 1.61 |
| Tryptophan | 1.89 | 2.52 | 2.55 | 1.44 | 1.86 |
| Leucine | 1.97 | 2.68 | 3.14 | 2.06 | 2.53 |

* See *Table 6

† All data are the average of 4 samples.

corresponding enzyme activities in M19 can be compared (Table 7). The rates of amino acid activation obtained by the exchange reaction versus that obtained by hydroxamate formation are not equivalent. The reason for this non-conformity is unknown, but it may be the result of the comparison of a biochemical reaction to one that is partially chemical in nature (Stulberg and Novelli, 1962). Though there is no inactivation of amino-acyl-t-RNA synthetase activity found at 30°C, the possibility that heat sensitivity in the esterification of amino acids to t-RNA, the interaction of the anti-codon site of t-RNA with the codon of the m-RNA of the ribosome and the peptide polymerization leading to synthesis of protein, cannot be excluded.

Malcolm (1967) also reported that protein synthesis and then, subsequently, RNA synthesis were halted after the cells of ATCC 15174 were transferred to 30°C in glutamate minimal medium. The cessation of protein synthesis was reported to occur after about 20 minutes and that of RNA after about 40 minutes following the shift to 30°C. However, when the incorporation of leucine -$^{14}$C into macromolecular protein was measured in the complex medium, protein synthesis continued for at least another 50 minutes at 30°C (Fig. 14) and more protein was synthesized than at 20°C. Malcolm (1967) observed that

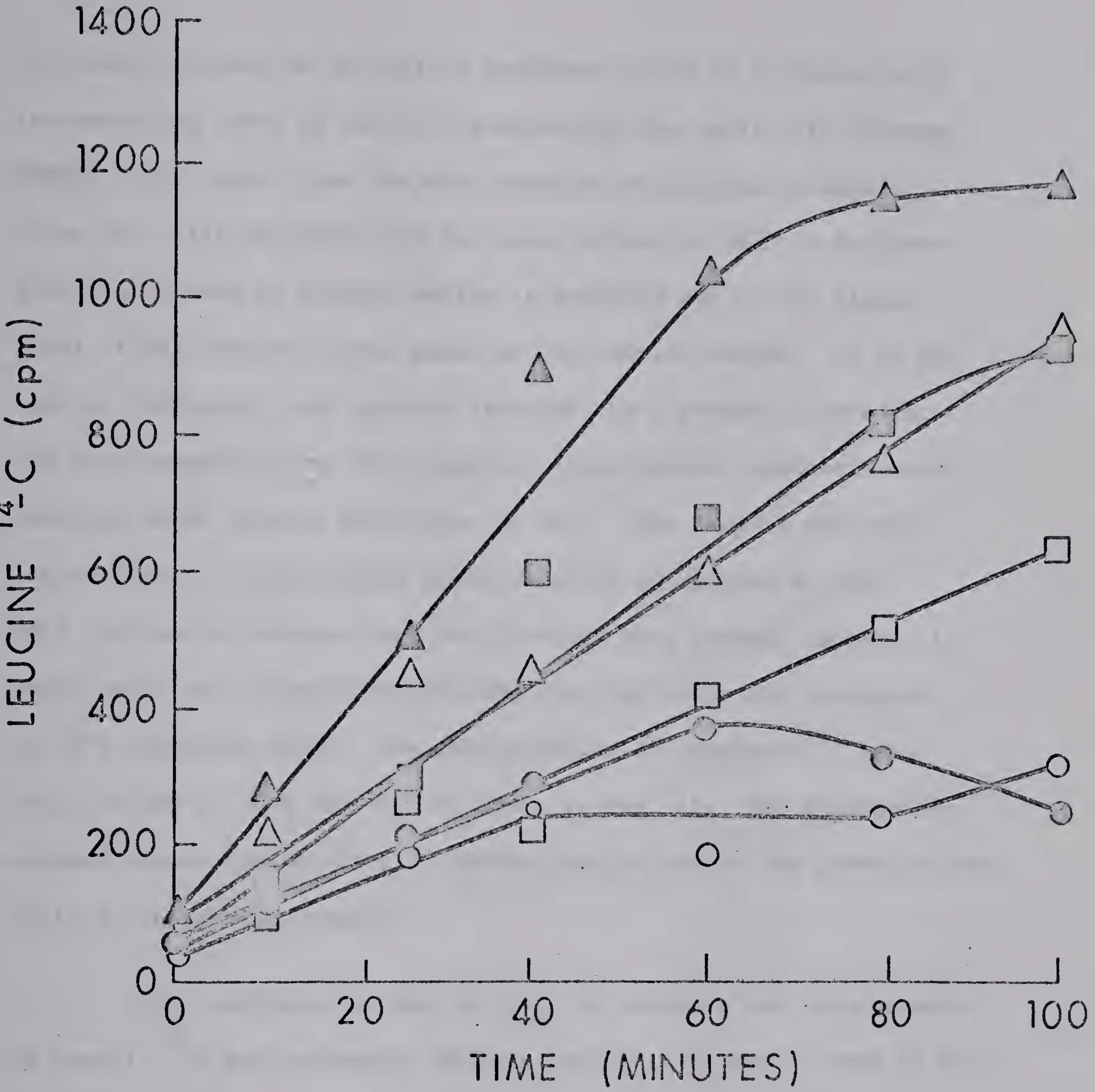

Fig. 14 Incorporation of Leucine-$^{14}$C into Macromolecular Protein and acid-soluble pool in ATCC 15174.

○——○ 20° C, acid-soluble
□——□ 20° C, acid insoluble protein
△——△ 20° C, uptake of leucine by cells (soluble + insoluble)
●——● 30° C, acid soluble
■——■ 30° C, acid-insoluble protein
▲——▲ 30° C, uptake of leucine by cells (soluble + insoluble)

the time of cessation of protein synthesis could be prolonged with increased RNA level of cells by pretreating the cells with chloramphenicol. The longer time for the cessation of protein synthesis after the cells of ATCC 15174 has been shifted to 30°C in the complex medium than in minimal medium is probably due to the higher level of RNA when cells are grown in the complex medium. It is unlikely, therefore, that protein synthesis is a primary factor for the heat-sensitivity of the organism, since protein synthesis could continue under certain conditions at 30°C. The drop of the radioactive level of acid-soluble fraction after 60 minutes at 30°C (Fig. 14) may be related with the findings that certain amounts of amino acids are released into medium when the cells are subjected to 30°C (Malcolm, 1967). The incorporation of leucine -$^{14}$C into cells of M19 at 20°C and 30°C is shown in Fig. 15. The sharply increased incorporation after 80 minutes may be due to the growth of the cells at those temperatures.

The synthesis of RNA in vivo by assaying the incorporation of uracil -$^{14}$C into polymeric RNA was studied. As can be seen in Fig. 16, the rate of macromolecular RNA synthesis in ATCC 15174 was reduced greatly after about 40 minutes at 30°C. The much greater rate of RNA synthesis after 80 minutes at 20°C may be due to the growth of the cells. Though there is only a slight difference in total

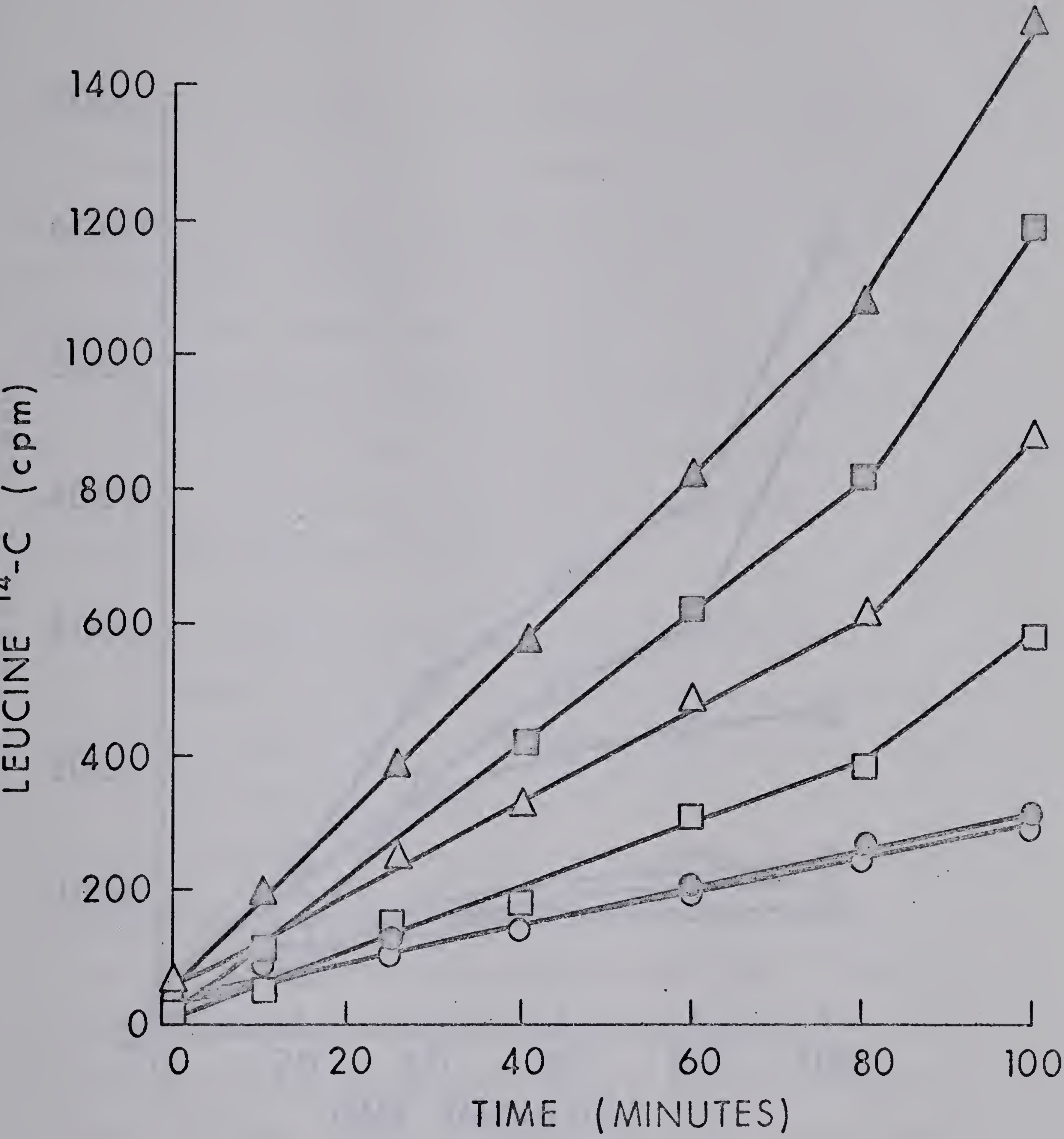

Fig. 15 Incorporation of Leucine - $^{14}$C into Macromolecular Protein and Acid-Soluble Pool in M19

○——○ 20° C, acid - soluble
□——□ 20° C, acid - insoluble protein
△——△ 20° C, uptake of leucine by cells (soluble + insoluble

●——● 30° C, acid - soluble
■——■ 30° C, acid - insoluble
▲——▲ 30° C, uptake of leucine by cells (soluble + insoluble

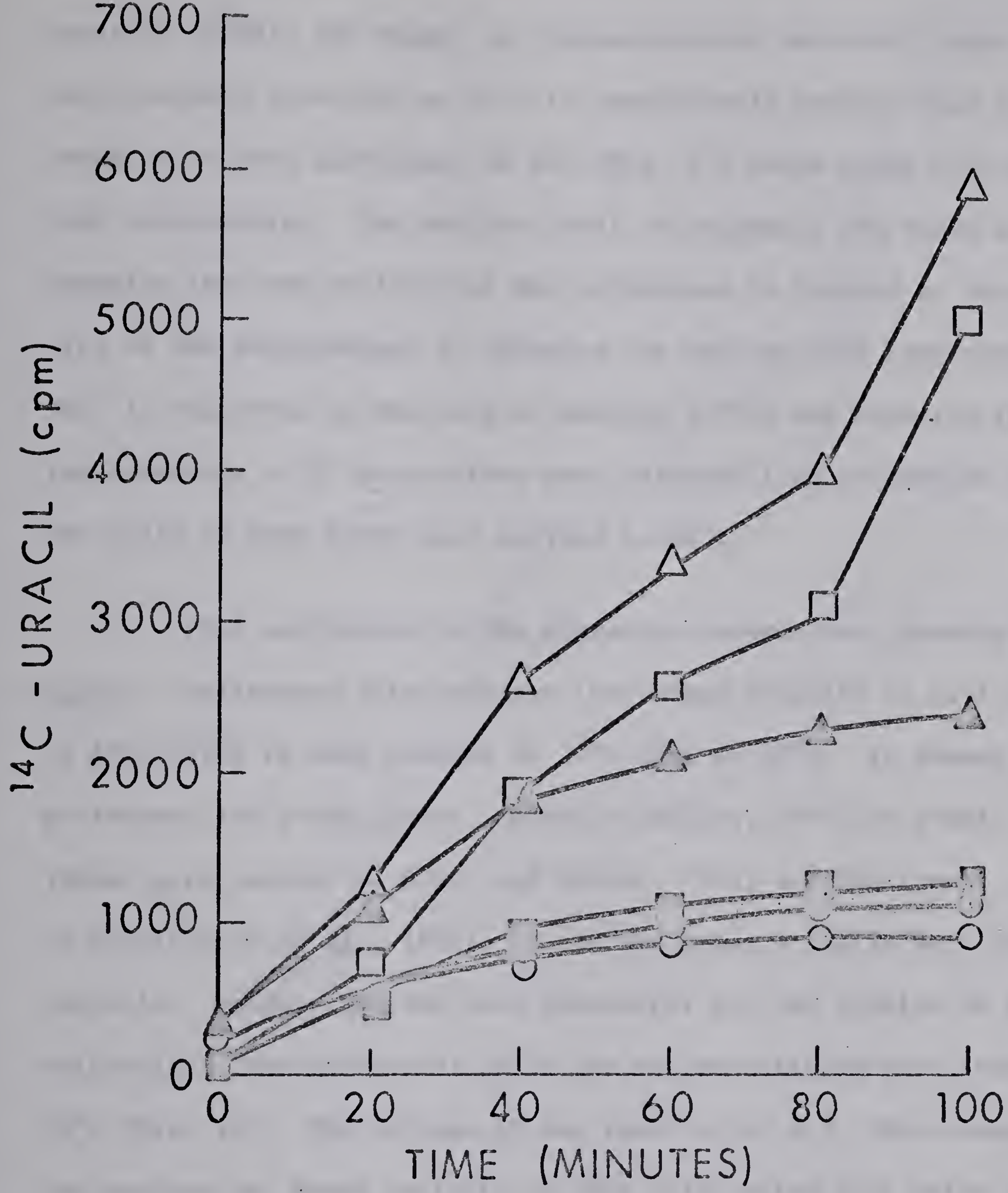

Fig. 16 Incorporation of Uracil - $^{14}$C into RNA and Acid-Soluble materials in ATCC 15174

○———○ acid-soluble fraction, 20°C
□———□ incorporation into RNA, 20°C
△———△ uptake of $^{14}$C-uracil by cells, 20°C (soluble + insoluble fractions)

●———● acid soluble fraction 30°C
■———■ incorporation into RNA, 30°C
▲———▲ uptake of $^{14}$C-uracil by cells, 30°C (soluble + insoluble fractions)

quantity of RNA, the amount of ribonucleotides and short chain RNA (acid-soluble fraction) at 30°C is consistently greater than at 20°C. There is no such difference in M19 (Fig. 17) which grows well at both temperatures. The smaller level of polymeric RNA found at 30°C suggests that the activity of RNA polymerase is reduced or the activity of RNA depolymerase is enhanced, or both, at this temperature. This is supported by the data of Malcolm (1967) who reported that large amounts of 5'-nucleotides were released into the medium after the cells of ATCC 15174 were shifted to 30°C.

The activities of RNA degrading enzymes were studied _in vitro_. Preliminary data indicate that RNase activity in cell extracts of ATCC 15174 is much greater at 30°C than at 20°C. It showed little preference for s-RNA (yeast, method of Holley, 1963) or r-RNA (wheat germ, method of Glitz and Dekker, 1963) and RNA (yeast, method of Crestifield _et al_., 1955), the former being a little more susceptible. Soluble RNA was used thereafter for the studies of RNase activities. The effects of pH on the enzyme activity were studied at 30°C (Fig. 18). The optimum pH was found to be 8.8. When assayed at the optimum pH, RNase activity of ATCC 15174 below 25°C which approximates to the maximal growth temperature of the organism was only slightly increased (Table 8). However, the activity increased

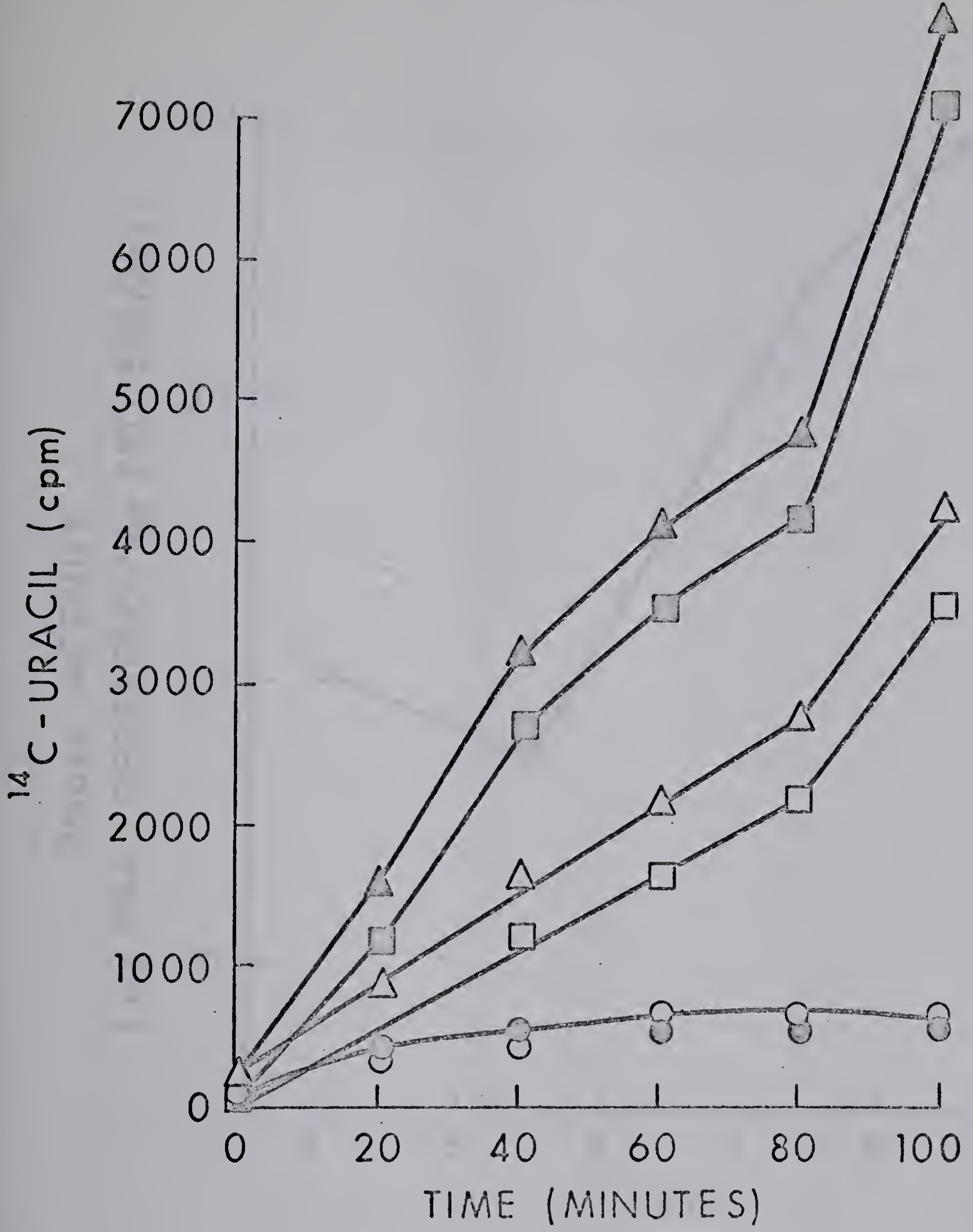

Fig. 17 Incorporation of Uracil - $^{14}$C into RNA and Acid-Soluble fraction in M19

○————○ acid-soluble fraction, 20° C
□————□ incorporation into RNA, 20° C
△————△ net uptake (acid-soluble + insoluble fractions) 20° C
●————● acid-soluble fraction, 30° C
■————■ incorporation in RNA, 30° C
▲————▲ net uptake (acid-soluble + insoluble fractions) 30° C

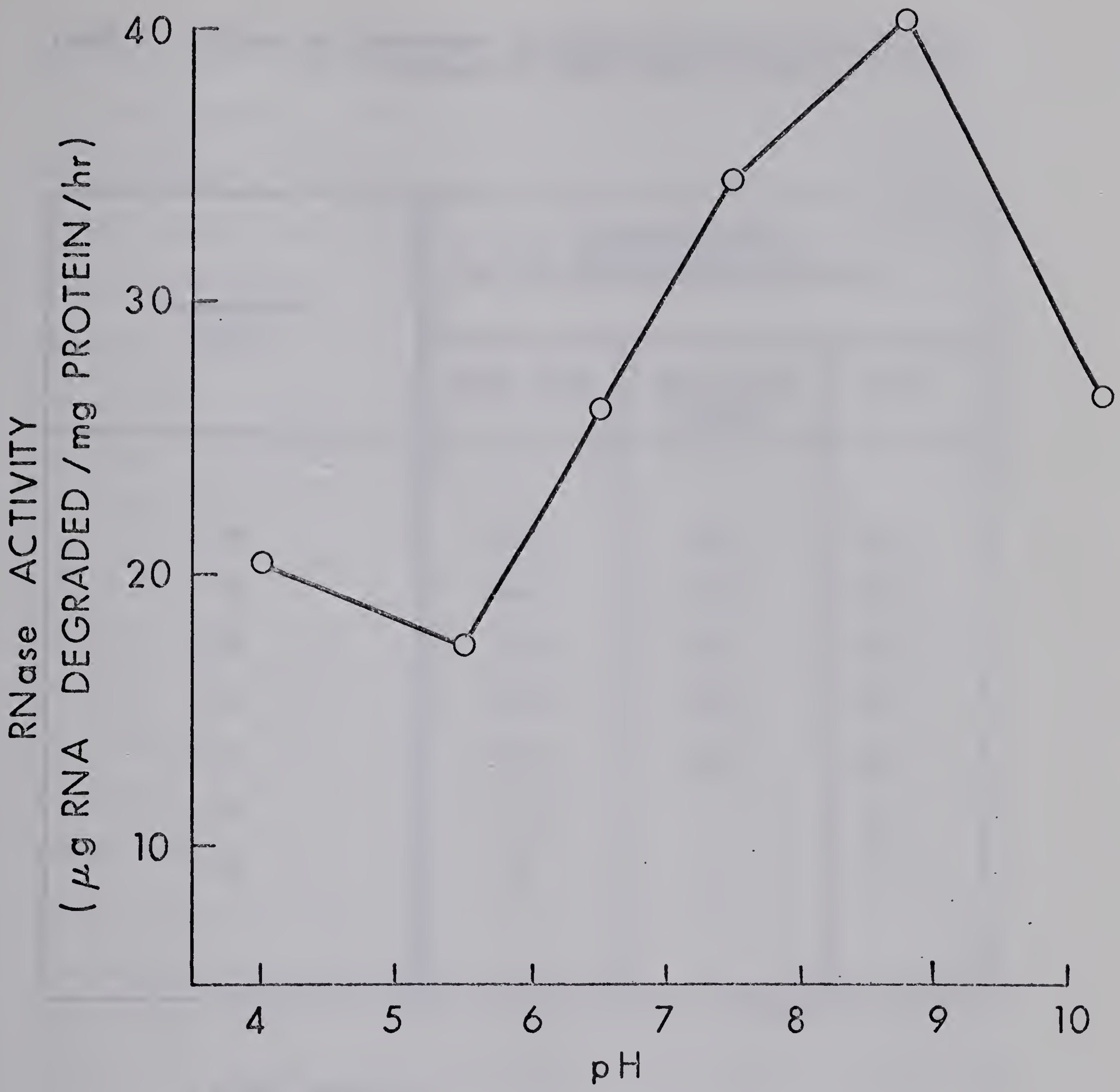

Fig. 18 Effect of pH on RNase activity of cell-free extracts of ATCC 15174 at 30° C

TABLE 8 Effect of Temperature on Ribonuclease Activity in Cell Extracts of ATCC 15174 and M19.†

| Temperature (°C) | Enzyme Activity ($\mu$g RNA degraded/mg protein/hr.) | | |
|---|---|---|---|
| | ATCC 15174 | ATCC 15174* heated | M19 |
| 20 | 23.1 | 28.7 | 30.2 |
| 25 | 24.4 | 34.9 | 31.6 |
| 30 | 43.9 | 46.1 | 32.3 |
| 37 | 50.1 | 52.4 | 34.7 |
| 45 | 21.5 | 20.4 | 56.3 |
| 50 | 4.2 | - | 15.2 |
| 55 | 0 | - | 1.1 |

* See *Table 6

† For original data see Appendix I, p. 146.

greatly at 30°C, almost doubling, and had a maximal activity at 37°C while RNase activity in M19 was almost constant at these temperatures. This phenomenon may indicate the turnover of RNA functions normally below 25°C, but above this temperature the RNase activity increases greatly resulting in breakdown of functional RNA in ATCC 15174. Once the enzyme has been heated at 30°C, it has a higher activity than that without the treatment at temperatures below 30°C, suggesting the "heat treatment" enhances the degrading activity, probably due to the change of structural moiety of the enzyme (Table 8). All the enzyme activities measured may be the net result of the activity of RNA polymerase and RNase. P-chloro-mercuribenzoate (PCMB) has been reported to inhibit RNA polymerase activity completely with a concentration of $10^{-4}$M (Hurwitz, 1963), while it has no effect on RNase even at $10^{-2}$M (Nishimura, 1960). The addition of PCMB increased the amount of RNA degraded (Table 9) and, again, RNase activity increased as the temperature increased above 25°C. However, PCMB seems to reduce RNase activity at higher temperature, i.e. at 45°C. It is interesting that RNase activity in cell extracts of M19 is nearly constant below 37°C, which is the maximal growth temperature of the organism, and increased greatly at 45°C (Table 8).

The effects of temperature on DNA-dependent RNA polymerase activity in ATCC 15174 are shown in Table 10. It was found that RNA

TABLE 9 Effect of p-chloromercuribenzoate (PCMB) on Ribonuclease Activity in Cell Extracts of ATCC 15174 at Different Temperatures.†

| Temperature (°C) | Specific activity ($\mu$g RNA degraded/mg protein/hr.) | | |
|---|---|---|---|
| | ATCC 15174 | ATCC 15174 + PCMB | Effect of PCMB |
| 20 | 23.1 | 27.3 | +4.2 |
| 25 | 24.4 | 27.5 | +3.1 |
| 30 | 43.9 | 45.8 | +1.9 |
| 37 | 50.1 | 50.1 | 0 |
| 45 | 21.5 | 12.5 | -9 |
| 50 | 4.2 | - | |

---

† For original data see Appendix I, p. 146.

TABLE 10 Effect of Temperature on RNA Polymerase Activity in Cell Extracts of ATCC 15174.†

| Assay Temperature (°C) | RNA polymerase activity (millimicromole ATP-$^{14}$C incorporated) ( /mg protein/hr. ) |
|---|---|
| 15 | 0.41 |
| 20 | 1.00 |
| 25 | 0.82 |
| 30 | 0.34 |
| 37 | 0.29 |

† All data are the average of 4 samples.

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polymerase had decreased synthesizing activity as the temperature increased above 20°C. The use of RNA as primer instead of DNA did not increase the activity. In view of the low enzyme activity of the cell extract this finding should be interpreted cautiously. Indirect support of this phenomenon comes from the data on the effects of PCMB on RNase activity. As indicated in Table 9, the increase of RNase activity upon the addition of PCMB decreases as temperature increases. This suggests that RNA polymerase activity decreases as temperature increases. However, the difference is only slight and therefore the reliability may be questioned.

The effects of temperature on DNase activity in the cell extract of ATCC 15174 and M19 were studied. As shown in Table 11, DNase activity in ATCC 15174 is about double at 30°C than at 20°C, though there is no such difference in M19.

## IV. ISOLATION AND CHARACTERIZATION OF RNASE AND RNA POLYMERASE

The results on the isolation of RNase are shown in Table 12. It can be seen that RNase was purified about 120-fold. The 280/260 ratio of the enzyme preparation was about 1.0, indicating about 3% of nucleic acids present (Layne, 1957). No further

TABLE 11 Effect of Temperature on Deoxyribonuclease in Cell Extracts of ATCC 15174 and M19.[†]

| Temperature (°C) | Enzyme activity (units/mg protein/hr.)* | |
|---|---|---|
| | ATCC 15174 | M19 |
| 20 | 10.3 | 9.6 |
| 25 | 13.1 | 9.0 |
| 30 | 25.0 | 9.8 |
| 37 | 18.3 | 11.0 |

* 1 unit is the enzyme activity that causes an increase of OD 0.01 at 260 mμ.

† All data are the average of two samples.

TABLE 12 Purification of *M. cryophilus* ATCC 15174 RNase.

| Fraction | Step | Total protein (mg) | Specific activity ($\mu$g RNA degraded /mg protein/hr.) | Relative purification | Total activity $\mu$g RNA (degraded/hr.) | Enzyme recovery (%) |
|---|---|---|---|---|---|---|
| I | Cell extracts | 1300 | 41.1 | 1 | 53430 | 100 |
| II | Protamine eluate | 57.6 | 372.3 | 9x | 21074.5 | 39.6 |
| III | Ammonium sulphate | 11.4 | 741.6 | 18x | 84542.4 | 15.6 |
| IV | DEAE-cellulose | 1.16 | 4960.4 | 121x | 5753.4 | 10.8 |

attempt was made to remove the contaminating nucleic acids.

The effect of pH on the activity of the purified RNase is shown in Fig. 19. It can be seen that the pH range is wider than that of the enzyme in cell extracts. However, the optimum pH 8.8 is the same in both. The effect of temperature on the activity at pH 8.8 is shown in Fig. 20. The results agree with the previous data of RNase activity in cell extracts that the activity is nearly constant below 25°C and increases greatly above this temperature and up to 37°C. The purified RNase lost about 20° activity after incubation at 45°C for 30 minutes, and was completely inactivated at 70°C for the same period of time (Fig. 21).

The activity of RNA polymerase after DEAE-cellulose fraction was not significantly increased, though protein content was reduced and therefore specific activity increased. The failure may be due to the insensitive method of assay. A knowledge of properties of RNA polymerase activities, especially the method of assay, is necessary before any further attempt to purify the enzyme can be made.

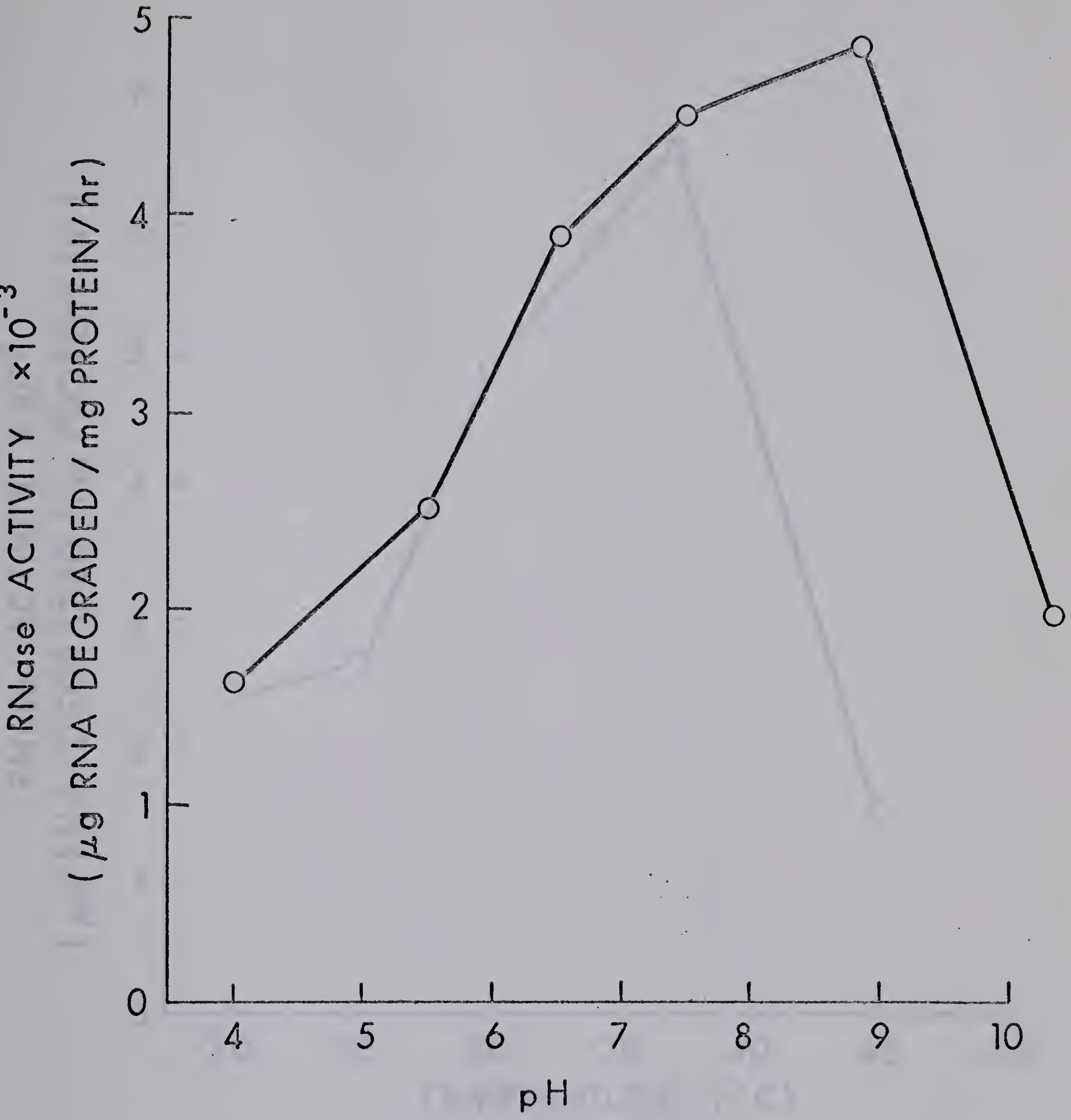

Fig. 19 Effect of pH on the Activity of Purified RNase

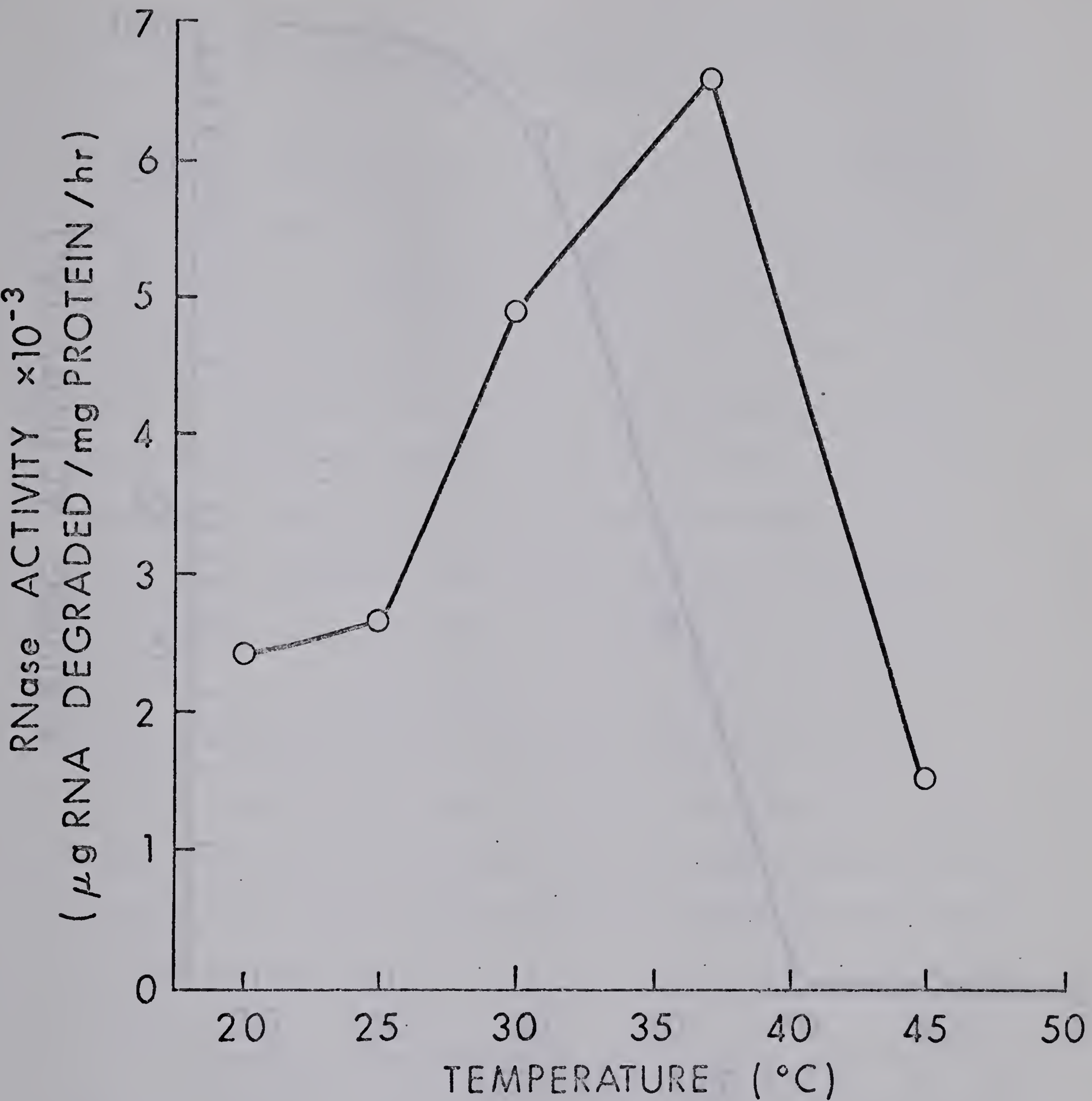

Fig. 20 Effect of Assay Temperature on Activity of Purified RNase

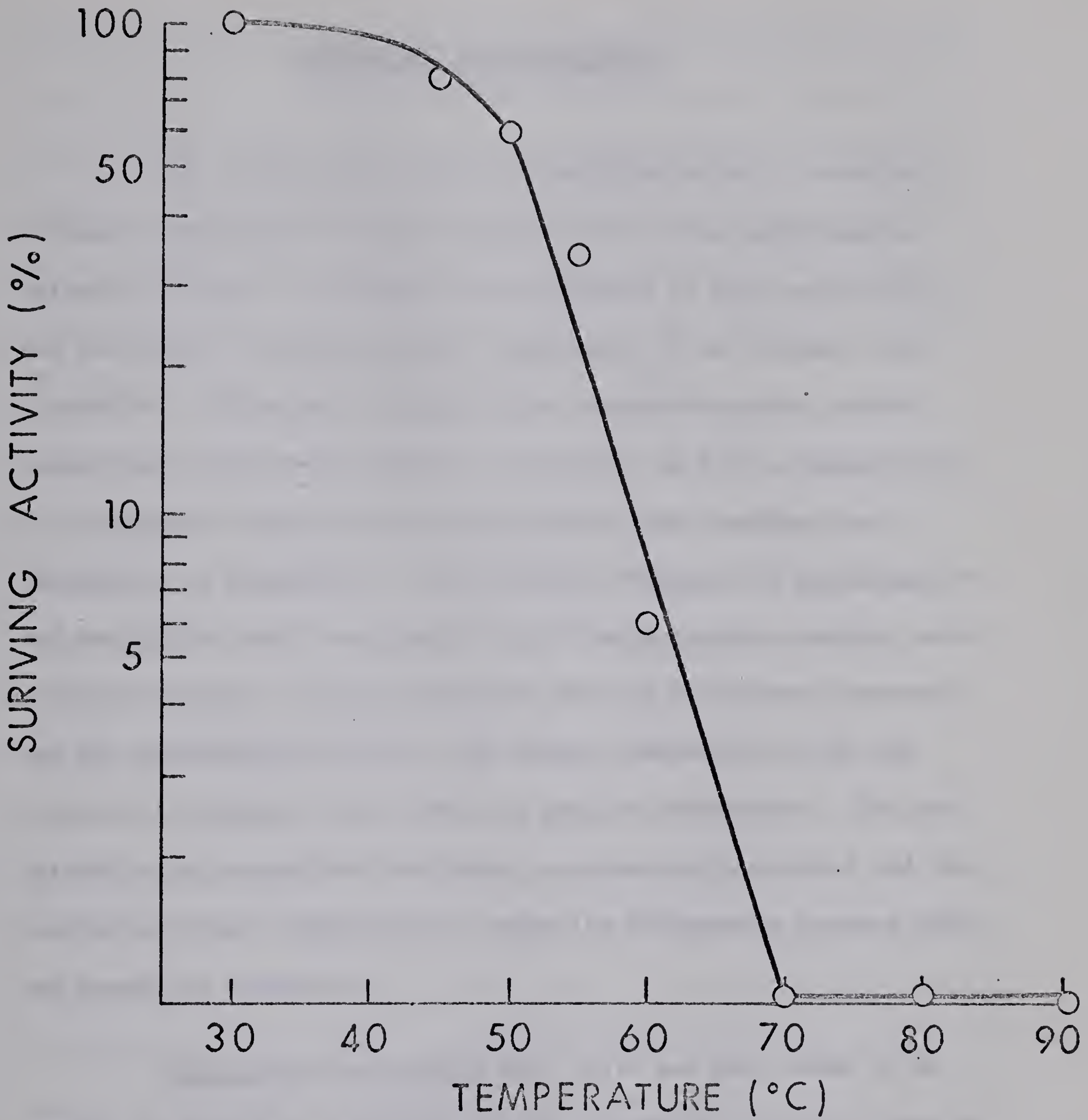

Fig.21 Heat Inactivation of Activity of Purified RNase

## DISCUSSION AND CONCLUSION

The primary aim of this investigation was to study an obligate psychrophilic micro-organism and its non-psychrophilic mutants in order to elucidate the phenomenon of heat-sensitivity and the basis of maximal growth temperature of an obligate psychrophile. The mutants obtained with increased maximal growth temperature lost their ability to grow well at 0°C, a characteristic considered essential for psychrophiles and therefore are designated as mesophiles. The different responses of psychrophiles and mesophiles have been compared previously by other workers using different species of micro-organisms and the differences observed may not necessarily be due to the unique characters of the two classes of organisms, but rather to species differences. The psychrophile and mesophiles used here are genetically related and thus provide an ideal combination to study the differences between these two groups of organisms.

Micrococcus cryophilus ATCC 15174 has been shown to be capable of growth on a minimal medium containing L-glutamic acid as sole carbon and nitrogen source. It obtains energy from glutamate via transaminase and aspartase to enter Kreb's cycle (Malcolm, 1967).

With 24 carbohydrates tested none has been fermented with the production of acid. Other observations agree with the results of McLean *et al*. (1951) except that *M. cryophilus* ATCC 15174 produces oxidase and has an optimum growth temperature, in terms of growth rate, at 20°C; the latter fits the observation that the maximum growth temperature is only a few degrees higher than the optimum growth temperature (Ingraham and Stokes, 1959).

Adaption of mesophiles to grow at temperatures above their maximum for growth has been reported and reviewed by Allen (1953). Such an adaption is also claimed recently (Brock, 1967). However, preliminary attempts to adapt *M. cryophilus* ATCC 15174 to grow at higher temperature by serial transfer with nutritional supplement were unsuccessful. Most mutants of micro-organisms with the ability to grow over a different temperature range from that of the parent by using mutagens are temperature-sensitive (see Introduction, Section IV) or cold-sensitive (O'Donovan *et al*., 1965; O'Donovan and Ingraham, 1965), and thus have a different range of temperature for growth. Condon and Ingraham isolated a mesophilic mutant from a psychrophilic pseudomonad for studies of minimal growth temperature (see Farrell and Rose, 1967). Azuma *et al*. (1962) were the first to claim the success of isolating

mutants with decreased minimal growth temperature. There is no report on the isolation of mutants with increased maximum growth temperature. With UV-irradiation, we were able to isolate some mutants with increased maximum growth temperature. Thus, the production of mutant strains of micro-organisms with the ability to grow over a different temperature range is established.

Transformation studies have been extended widely, many cases of interspecific and even intergeneric transformation have been reported (Schaeffer, 1963). McDonald and Matney (1963) were the first to report the interesting transformation of thermophilic transformants capable of growth at 55°C from a mesophilic strain of Bacillus subtilis by treating DNA preparation from a facultative thermophile. The character controlling the ability appears, as claimed, to be closely linked to the single-step high level streptomycin-resistant locus. Further investigation has been reported (McDonald, 1967). The genetic transfer of growth temperature is confirmed from the isolation of transformants at 30°C, by applying DNA preparation of mesophilic mutants to M. cryophilus ATCC 15174 cultures which failed to grow above 24°C. However, the genetic transfer of this character seems to be a more complicated phenomenon than that of a single mutation such as sporulation,

drug resistance or capsule formation, since most transformants subsequently lost their ability to grow at 30°C after replication, and therefore represented merely phenotypic transformation. The possibility of protection of DNA polymers responsible for phenotypic expression has been ruled out by using controls which did not bring about the formation of colonies at 30°C. The mechanism of the transformation and the reasons why up to 90% of the transformants lost the ability to grow at 30°C after multiplying for several generations to form a visible colony are unknown. However, Hurwitz *et al*. (1963) reported the temporary phenotypic transformation related to RNA polymerase function, in which transforming DNA can be used for the *in vitro* synthesis of RNA and caused a phenotypic transformation of pneumococci after uptake of such RNA.

Two mutants, T8 and M19, with increased maximum temperature of growth have been isolated from *M.cryophilus* ATCC 15174 and shown to be genetically related to the wild type by transformation studies. These two mutants possess most of the general characters of the parent. However, the mutants grow much more slowly in glutamate minimal medium than the parent but much better in TSB medium at higher temperatures. This observation combined with the findings of respiratory studies, Kreb's cycle enzyme activities,

and oxidase and nitrate reduction ability, indicate the change of metabolic pathway of mutants. The decreased activity of transaminases, at least glutamate-oxaloacetate transaminase, may be responsible for the poor growth of the mutants in the glutamate minimal medium. Increased nutritional requirements of micro-organisms to grow at a new range of temperature are not uncommon (Ingraham, 1962). It is understandable that mutants have different metabolic pathways and require some nutrients in addition to, or replacing, glutamate to support better growth, while endowed with the ability to multiply at elevated temperature. With such an understanding, no particular attempts to study nutritional requirements for mutants nor why they grow poorly in glutamate minimal medium were carried out.

Gause *et al*. (1967) reported the isolation of mutants with altered DNA base composition. This is not the case in mutant M19. DNA of ATCC 15174 contains about 37% Guanine-Cytosine, while M19 about 38%, near that reported for *M. cryophilus* (Marmur et al., 1963) and only slightly different from that reported by Bohacek *et al*. (1967) for an organism designated as *M. cryophilus* (pediococcus). The G-C content in DNA of *M. cryophilus* shows a marked difference from micrococci which, in general, have a much higher G-C content

(65.6 - 75.5%). This supports the claims that M. cryophilus should be removed from the genus Micrococcus (Marmur et al., 1963; Bohacek et al., 1967). Denaturation temperature of DNA in ATCC 15174 84.5°C) and M19 (85°C) shows little relation to maximum growth temperature as indicated by Marmur (1961). Though the mutants have higher thermal death point (TDP) than the parent, heat-sensitivity is only slightly related to maximum growth temperature (MGT), since mutant T8 with a MGT at 34°C is more heat-resistant than M19 with a MGT at 37°C, in terms of both TDP and D values.

Mutants T8 and M19 fail to form colonies on agar plates at 0°C, and though they are able to grow at 0°C in broth medium with a generation time of about 55 hours, they should be excluded from the group of psychrophiles. They may be better designated as mesophiles. Psychrophilic ATCC 15174 has a temperature characteristic ($\mu$) of about 10,100 calories in the temperature range of 8°C to 20°C, while mesophilic T8 and M19 about 16,100 and 15,000 respectively. Ingraham (1958) previously reported the difference of temperature characteristic, $\mu$, between a psychrophilic pseudomonad and a mesophilic E. coli and suggested that psychrophiles may be characterized by their lower $\mu$ value than mesophiles. The results obtained here seem to support Ingraham's suggestion that psychro-

philes have lower $\mu$ values than mesophiles, using a psychrophile and its mesophilic mutants, and hence eliminating species differences. However, Shaw (1967), and Hanus and Morita (1967) both report that the psychrophiles cannot be distinguished from the mesophiles on the basis of the temperature characteristic. In view of these different findings it would appear reasonable to suggest that the temperature characteristic for different species of organisms may be the same, regardless of their growth range, however, if two organisms are derived from the same strain and hence very closely related genetically, $\mu$ value may be used to differentiate between them; the organism with a higher minimum growth temperature having a higher $\mu$ value than that with a lower minimum growth temperature. Support for this suggestion can be found from the data of O'Donovan _et_ _al_. (1965) and Ingraham and Marr (1965) who compared a cold-sensitive mutant K-I with the parent, _E_. _coli_ C-600-1. However, a conditional mutant which requires histidine only at low temperature (O'Donovan _et_ _al_., 1965), has a same $\mu$ value in complex medium and minimum medium supplemented with histidine as its parent and the above suggestion is correct only when mutant K-II-27 and its parent C-600-1 were grown in minimal medium. Therefore, more detailed studies should be made before one makes use of the term $\mu$ to characterize groups of organisms on the

basis of their temperature relationships.

Baxter and Gibbons (1962) found that a psychrophilic strain of Candida respired endogenous reserves at a greater rate than the mesophile at all temperatures up to 30°C. This is not the case in this study. More likely, endogenous respiration is not a factor for the differences between psychrophiles and mesophiles. Nor would $QO_2$ of an oxidizable substrate appear to be a factor since this reflects the ability of an organism to obtain energy from a substrate, a change of substrate would vary $QO_2$ values. $Q_{10}$ may be a better expression for the differences due to the changes in temperatures. Brown (1957) reported that the oxygen uptake with glucose by a psychrophile had a lower $\mu$ than that of mesophilic Ps. aeruginosa, and showed that the psychrophile was less sensitive to changes in temperature. This is unlikely to be a common difference between psychrophiles and mesophiles. ATCC 15174 showed a sharper increase of $QO_2$ below 20°C and a greater decrease of $QO_2$ above 20°C than the mutants for the oxidation of glutamate. If an Arrhenius plot of rates of glutamate oxidation were made, one would expect to obtain a higher value of $\mu$ for ATCC 15174 between 10 to 20°C. This should not be confused with $\mu$ of growth rate. Growth rate is a total expression of the reactions by an organism at a given temperature while oxygen uptake is merely an energy-obtaining ability at that temperature.

ATCC 15174 has a much greater $Q_{10}$ (10 - 20°C) as compared to mutants, corresponding to the lower activation energy ($\mu$) required during the temperature range of growth. It is reasonable that ATCC 15174 which requires less activation energy to grow has a higher $Q_{10}$, obtaining more energy from the substrate, and therefore grows better than mutants between 10 to 20°C. Above the maximum growth temperature, $\mu$ of growth of ATCC 15174 becomes infinite and cells incapable of growth result, while $Q_{10}$ values drop sharply. If the total response of $Q_{10}$ is accepted with the reference temperatures set at 10°C and 30°C (Ingraham and Bailey, 1959), both mutants T8 and M19 have a higher $Q_{10}$ of 2.1 while the psychrophilic parent is about 1.3, only two-thirds of that of mesophiles, a difference that has been observed by Brown (1957) and Ingraham and Bailey (1959), applying various oxidation systems. As the use of temperature characteristic of growth of organisms to differentiate mesophiles and psychrophiles is being questioned, the term $Q_{10}$ of substrate oxidation, with reference temperatures set at 10 and 30°C, may well be a better parameter to characterize these two classes of organisms.

Malcolm (1967) has shown that heat-sensitivity of *M. cryophilus* ATCC 15174 is not due to the blocking of a specific

biosynthetic pathway or heat-induced damage of the cellular membrane leading to leakage of intracellular material, nor due to having the presence of heat-sensitive enzymes of Kreb's cycle. No evidence of any temperature-sensitivity in respiration solutes-transporting and energy-obtaining system has been found. The decrease of rate of glutamate oxidation at 30°C in this organism is not affected by protein synthesis as assayed with the addition of chloramphenicol at 20°C and 30°C, but rather by a change of cellular organization at 30°C, though this is not necessarily the primary effect of exposure to moderate temperature.

The results on the temperature-sensitivity of amino acyl-t-RNA synthetases differ from those of Malcolm (1967). Malcolm, using the hydroxamate method found that of 18 amino acids studied, 6 had reduced activities at 30°C. As all of the activities reported were very low, this experiment was repeated using the hydroxamate method and the much more sensitive pyrophosphate exchange method. The 6 sensitive enzymes reported by Malcolm together with leucine were used. No inactivation of the amino acyl-t-RNA synthetases was detectable at 30°C. In most cases the activities at 30°C were, in fact, higher than at 20°C. The duration of the actual experimental procedure was 30 minutes. The possibility that this period of time is insufficient

for inactivation was eliminated by the use of control cells previously subjected to 30°C for 6 hours before disruption of the cells. The experiment was repeated using M19, the mesophilic mutant, and again no inactivation of enzyme activity was observed. From these results it is concluded that the heat-sensitivity of ATCC 15174 is not due to inactivation of amino acyl-t-RNA synthetases, leading to cessation of protein synthesis. The possibility of heat-sensitivity in the subsequent processes, i.e. esterification to t-RNA, the interaction of the anti-codon site of t-RNA with the codon of the m-RNA of the ribosome and the peptide polymerization cannot be excluded.

Studies on incorporation of $C^{14}$-leucine in ATCC 15174 at 30°C showed that protein synthesis ceased after approximately 80 minutes. This differs from the results of Malcolm (1967) who reported that protein synthesis ceased after 15 to 20 minutes. It is important to note that the medium used in this experiment was complex medium, whereas Malcolm used minimal medium. This difference in media could account for the differences in time for cessation of protein synthesis. On transfer to 30°C the continuation of protein synthesis is dependent on the level of RNA in the cells at the time of transfer. It would be expected that the level of

RNA in cells grown on complex medium would be higher than in those grown on minimal medium, and as a result protein synthesis could continue for a longer period of time in the former. This is supported by the findings of Schaechter et al. (1958) who showed that the RNA content of cells was greatly affected by differences in the composition of media. It has also been shown that the faster cells grow the richer they are in RNA (Brachet, 1955, Kurland and Maaløe 1962). If the above suggestions are correct the treatment of cells with chloramphenicol prior to exposure at 30°C should affect the time for cessation of protein synthesis, as a result of the accumulation of RNA. This was shown by Malcolm, who found that exposure of cells to chloramphenicol for 10 minutes prior to transfer to 30°C did in fact increase the time to cessation of protein synthesis from 15 to 20 minutes to 45 minutes.

Since protein synthesis can be carried out under certain conditions at 30°C it would appear that the heat-sensitivity of protein synthesis is not the primary factor in the heat-sensitivity of ATCC 15174.

RNA synthesis is affected in ATCC 15174 after 40 minutes of exposure to 30°C, in minimum medium (Malcolm, 1967), as measured by incorporation of uracil-$^{14}$C into RNA. The results from this

study show that when complex medium is used the result is the same. At 30°C, the acid-soluble fractions are consistently, though only slightly, higher than at 20°C, and the amount of incorporated macromolecular RNA is greatly reduced at 30°C as compared with that at 20°C. No such differences could be found in the mesophilic mutant M19. These findings suggest both RNA polymerase and depolymerase are involved in heat-sensitivity and that the results arise from the net activity of both of these enzymes. The results are based on the assumption that uracil is incorporated only into RNA. This is not absolutely correct, since it has been shown that uracil can be incorporated into DNA (Richardson *et al.*, 1963). If it is assumed that incorporation of uracil into DNA is much slower than into RNA, then this fact becomes less important.

RNase in cell extracts of ATCC 15174 shows little preference for substrate between t-RNA (yeast) and r-RNA (wheat germ), the former being a little more susceptible. The optimum pH of the enzyme is 8.8, which is different from the intracellular RNase in *Bacillus subtilis* with pH 5.8 as optimum (Nishimure and Maruo, 1960) while it is similar to extracellular nuclease of *M. sodonensis* (Berry and Campbell, 1967). A very interesting finding is that RNase activity is somewhat constant below 25°C, which is about the

maximum growth temperature of the organism, but increases rapidly above the temperature up to 37°C. RNase, 120-fold purified, from ATCC 15174 exhibits the same temperature response, whereas RNase activity in cell extracts of M19, does not. Therefore, RNase would appear to play an important role in the heat-sensitivity of ATCC 15174. The RNase activity may be greatly increased by the moderate temperature to 30°C. Cell extracts which have been heated at 30°C for 6 hours before the disruption of cells, exhibit a greater RNase activity than those without the treatment. Therefore, it is possible that RNase is activated and probably a structural change occurs in the enzyme molecules at higher temperature. However, the "activated" form of RNase may be recovered to near "native" form, since the increased activity of "activated" RNase decreases as the assay temperature decreases. It has been reported that ribonuclease II has different states and equilibrium between the native molecule state and sequential three-state denaturation (Sarfare and Bigelow, 1967). Various activities of RNase in different states of the enzyme would be expected. Though, there is no direct evidence of the action of RNase, ribosomes and r-RNA broken down due to heat treatment can be re-synthesized during the recovery of the synthesizing and degrading enzyme to the native forms (Sogin and Ordal, 1967).

The possibility that the greater amount of RNA broken down at high temperature is due to a conformational change of substrate itself making it more susceptible to the attack of RNase cannot be neglected. Sarin *et al*. (1966) indicated a difference of conformation in the secondary structure of several types of purified yeast t-RNA. The t-RNA underwent conformational changes between 22°C and 40°C. Therefore, rather than the enzyme activity itself being changed, alteration of the site of attachment on the t-RNA molecule for the RNase might be involved, or the RNA might alter the specificity of the RNase by changing its conformation (allosteric alteration) as reported by Loftfield and Eigner (1965) in the cases of certain amino acyl-t-RNA synthetases. Elson (1958, 1959) reported a ribonuclease tightly bound to 30S ribosomes in a latent form. Such an enzyme was also found by Stenesh and Yang (1967). No ribonuclease activity is present prior to ribosome break down but the enzyme is active in free form after ribosome break down (Spahr and Hollingworth, 1961). If RNase activity in ATCC 15174 is similar to the latter case, then why should RNase activity be much higher at 30°C than at 20°C, as the ribosomes are unlikely to be broken down at 30°C. The observations that RNase activity is higher in "activated" samples than in untreated ones at temperatures of 30°C and below may provide evidence to rule

out the configurational change of substrate leading to the greater amount of RNA break down at higher temperature. This is further supported by the data that partially purified RNase which has been "heated" at 37°C for 30 minutes shows greater activity than that "heated" at 30°C, both being assayed at 30°C. It would appear more likely that the greater RNase activities at higher temperatures is due to the activation of the enzyme at those temperatures, probably a change of structural configuration of enzyme molecules.

There is another possible mechanism that may lead to the higher RNase activity at higher temperature. If a regulatory system for RNase is present, then any factor bringing about a change in activity or affinity of active repressors (apo-repressor + co-repressor) would affect the RNase activity. A control system in which affinity of the inducer for the repressor protein is decreased at low temperature has been established and the regulatory system is suggested to be the direct cause of a minimal growth temperature (Marr _et_ _al_., 1964; O'Donovan _et_ _al_., 1965). The inhibition of the repressor activity may lead to the great increase in RNase activity. Under this condition, high RNase II activity after break down of bounded ribosomes (Spahr and Hollingworth, 1961; Spahr, 1964), degradation of r-RNA and ribosomes after heat exposure (Sogin and

Ordal, 1967; Stenesh and Yang, 1967) and degradation of ribosomes and RNA by the starvation of bacteria for thymine and amino acids (Doudney *et al.*, 1967) would be the expected results if the mentioned treatments alter or inhibit active repressor and therefore change their regulation activity on RNase. It is unlikely, however, that the increased RNase activity is due to the inhibition or altered affinity of repressor, since the activity of purified RNase increased with increasing temperature. However, it could be argued that the enzyme is only partially purified and that in this form the repressor is still bound or present in the enzyme preparation. The use of homogenous, highly purified RNase would be necessary to test this hypothesis.

DNA-dependent as well as RNA-dependent RNA polymerase activities *in vitro* were also studied. It was shown that RNA polymerase activity decreased as the temperature increased above 20°C, the optimum growth temperature of ATCC 15174. Actually, several trials have shown that RNA polymerase is completely inhibited at 30°C. Indirect support comes from data using an inhibitor, PCMB, to measure the RNase activity. Hara and Mitsu (1967) have shown that the decrease of DNA-dependent RNA polymerase activity in *E. coli* is due to the degradation of the enzyme itself. The findings in

this present work should be interpreted cautiously because the activities measured in cell extracts were very low. Attempts to purify the RNA polymerase were unsuccessful, probably due to the insensitive assay method applied. The properties of RNA polymerase in ATCC 15174 remain unclear.

Heat-sensitive DNA-synthesizing enzyme has been reported (Fangman, 1966). Hartwell (1967) observed that some temperature-sensitive mutants are due to the inability to carry out DNA synthesis at higher temperatures. DNA synthesis of ATCC 15174 *in vitro* was studied by measuring incorporation of thymine-$^{14}$C into macromolecular DNA. However, for some unknown reasons, the incorporation of thymine in the complex medium is very slow and only in a small amount at both 20°C and 30°C. *In vitro*, DNase activity in cell extracts is much greater at 30°C than at 20°C. It is not clear whether DNA and RNA degrading enzymes are one and the same or not, even though the DNase activity is optimal at 30°C while that for RNase is at 37°C. The assay of partially purified RNase on DNA as substrate suggests that they are probably different enzymes.

The results of this study point to the significance and importance of RNA and its associated enzymes and functions as a prime factor in relation to the heat-sensitivity of *M. cryophilus*.

In order to discuss the possible biological significance of these findings, it is necessary to consider the relationship of RNA and its associated enzymes to protein synthesis. It has long been established that the synthesis of protein requires three classes of RNA,namely, m-RNA, t-RNA and r-RNA in a certain proportion to function normally. A decrease of any class of functional RNA below a certain level would lead to cessation of protein synthesis. This has been demonstrated by Nihei and Cantoni (1963), and Zubay and Takanami (1964) who showed that when the level of t-RNA fell below 5% of normal, it lost the ability to accept activated amino acids and eventually a halt of protein synthesis would be expected. Byfield and Scherbaun (1967) reported that the destruction of template RNA led to the cessation of protein synthesis in a protozoan at temperatures above the optimum for growth. Thus, should any factor increase the rate of degradation or decease the rate of synthesis of any class of RNA, protein synthesis would eventually halt. This seems to be the case in _M_. _cryophilus_ ATCC 15174. As temperature increases, "activated" RNase breaks down more RNA. It is known that r-RNA comprises a higher percentage of total RNA than m-RNA and t-RNA (Ingram, 1965). Since we have shown that RNase degrades almost an equal amount of t-RNA, r-RNA and mixed RNA, the level and proportion of t-RNA would be affected

before r-RNA. In such a manner, even though RNA synthesis continues at 30°C and total RNA is maintained at a certain level, protein synthesis would be halted due to the lack of functional t-RNA, this, in turn, would affect RNA synthesis itself and cause the cessation of growth. The imbalance of the three classes of RNA may be the reason why the RNA synthesized at 30°C is unable to sustain protein synthesis as questioned by Malcolm (1967). The action of RNase on m-RNA is unknown, although it is possible that m-RNA is affected in same manner as t-RNA. Since RNA polymerase showed decreased activity at higher temperatures, possibly less functional RNA in the cells is synthesized. A change of the ratio of three classes of RNA synthesized due to the effect of elevated temperature is possible. A detailed analysis of the classes of RNA after cells are shifted to 30°C may provide the necessary information.

It is unknown at present whether "activated" RNase would lead to the fatality of the cells of _M_. _cryophilus_ at the elevated temperature. The death rates of cells at 30°C is known to be slower in the presence of more nutrients in the medium, and thus less pronounced in the complex medium than in minimal medium. Thompson and Shively (1966) treated a thermophilic bacillus with

pancreatic RNase and reported a drastic reduction of viable cells. An RNase attached to ribosomes would not be lethal to its host cells, however, if detached, it would become activated, and hence lethal (Spahr and Hollingworth, 1961; Spahr, 1964). It is quite possible that the "activated" RNase in M. cryophilus breaks down significant amounts of functional RNA and causes a disorganization of the cells at 30°C and leading to a loss of viability.

The possibility of DNase activity being involved in the heat-sensitivity of this organism cannot be excluded. The sequence is unknown whether the degradation of DNA causes the cessation of DNA dependent RNA polymerase activity or the unavailability of RNA halts the transcription of DNA information. The events may occur at the same time or they may be unrelated to each other during short time intervals, since both DNase and RNase activities are much greater at 30°C than at 20°C.

Much still has to be done on the heat-sensitivity and the basis of maximum growth temperature (MGT) of M. cryophilus ATCC 15174. However, from the findings of this work, it is probable that the enzymes associated with the synthesis and degration of RNA, and possibly those of DNA, play the most important role in determining

the heat-sensitivity of the organism. The action of RNase on functional RNA, likely t-RNA, is the most probable basis of MGT of _M. cryophilus_. In the mesophilic mutant M19, RNase activity is almost constant at and below 37°C, the MGT of this organism, and shows greatly increased activity at 45°C. The basis of MGT of the mutant may thus be the same as its parent.

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## APPENDIX I

Raw data for Tables 8 and 9.

| Temperature °C | Enzyme activity (as measured by O.D.) | | | |
|---|---|---|---|---|
| | ATCC 15174 | ATCC 15174 heated | ATCC 15174 + PCMB | M 19 |
| 20°C | 0.113 | 0.140 | 0.132 | 0.148 |
| | 0.114 | 0.141 | 0.132 | 0.150 |
| | 0.114 | 0.140 | 0.132 | |
| | 0.110 | | | |
| | 0.112 | | | |
| 25°C | 0.115 | 0.170 | 0.134 | 0.148 |
| | 0.114 | 0.151 | 0.136 | 0.154 |
| | 0.116 | 0.191 | 0.138 | |
| | 0.112 | | | |
| | 0.114 | | | |
| 30°C | 0.215 | 0.226 | 0.225 | 0.159 |
| | 0.216 | 0.228 | 0.226 | 0.161 |
| | 0.734 | 0.225 | 0.226 | |
| | 0.205 | | | |
| | 0.235 | | | |
| 37°C | 0.25 | 0.258 | 0.25 | 0.168 |
| | 0.252 | 0.255 | 0.253 | 0.166 |
| | 0.247 | 0.254 | 0.247 | |
| | 0.25 | | | |
| | 0.249 | | | |

## APPENDIX I (continued)

| Temperature °C | Enzyme activity (as measured by O.D.) | | | |
|---|---|---|---|---|
| | ATCC 15174 | ATCC 15174 heated | ATCC 15174 + PCMB | M 19 |
| 45°C | 0.106<br>0.107<br>0.104<br>0.118<br>0.115 | 0.115<br>0.105<br>0.105 | 0.060<br>0.070<br>0.060 | 0.260<br>0.260 |
| 50°C | 0.000<br>0.012 | | | 0.092<br>0.095 |
| 55°C | 0.000<br>0.000 | | | 0.000<br>0.005 |

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